Gene-editing and therapeutic applications

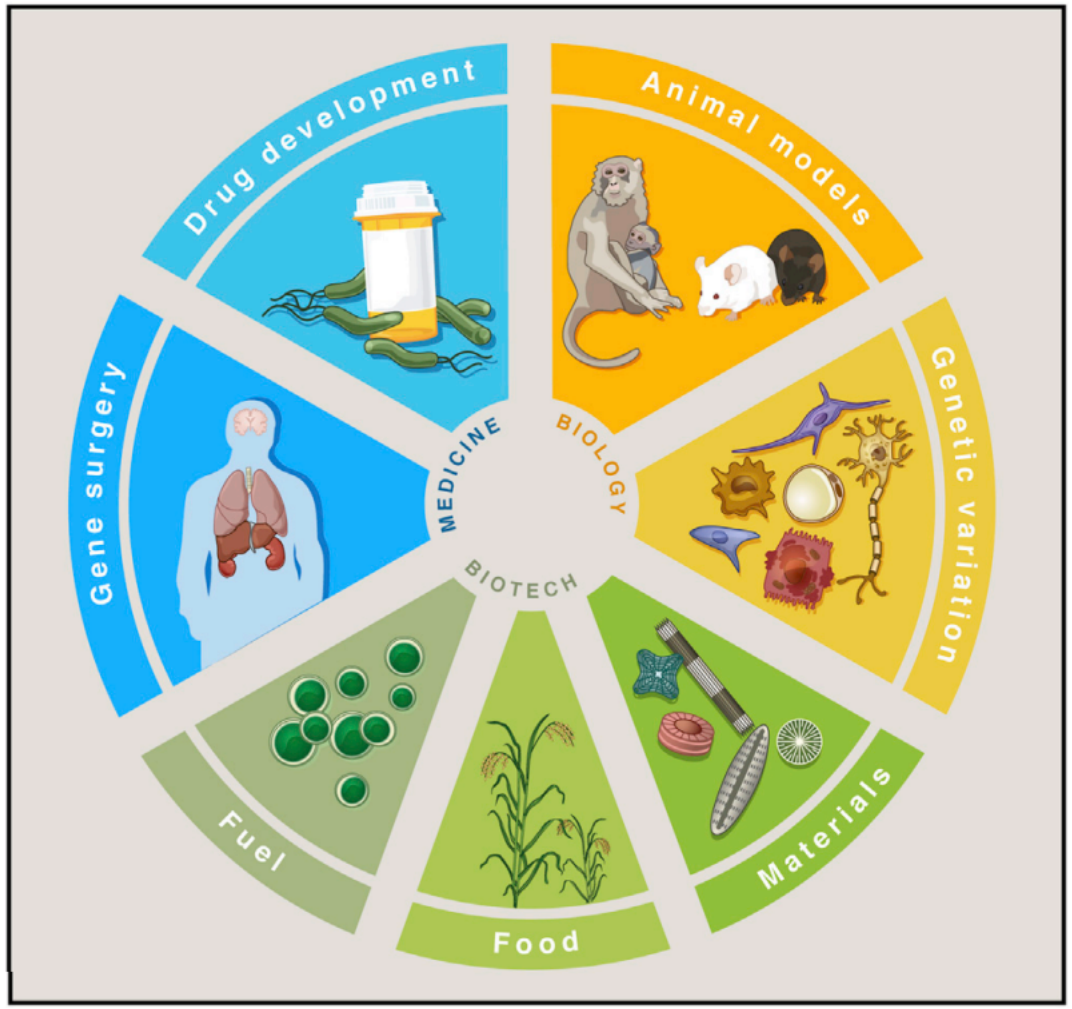
Dr Arkadiusz Kajdasz

Lecture plan

1. Genome engineering (gene therapy)
2. Methods of genome-editing
   - DNA double-stranded break (**DSB**)

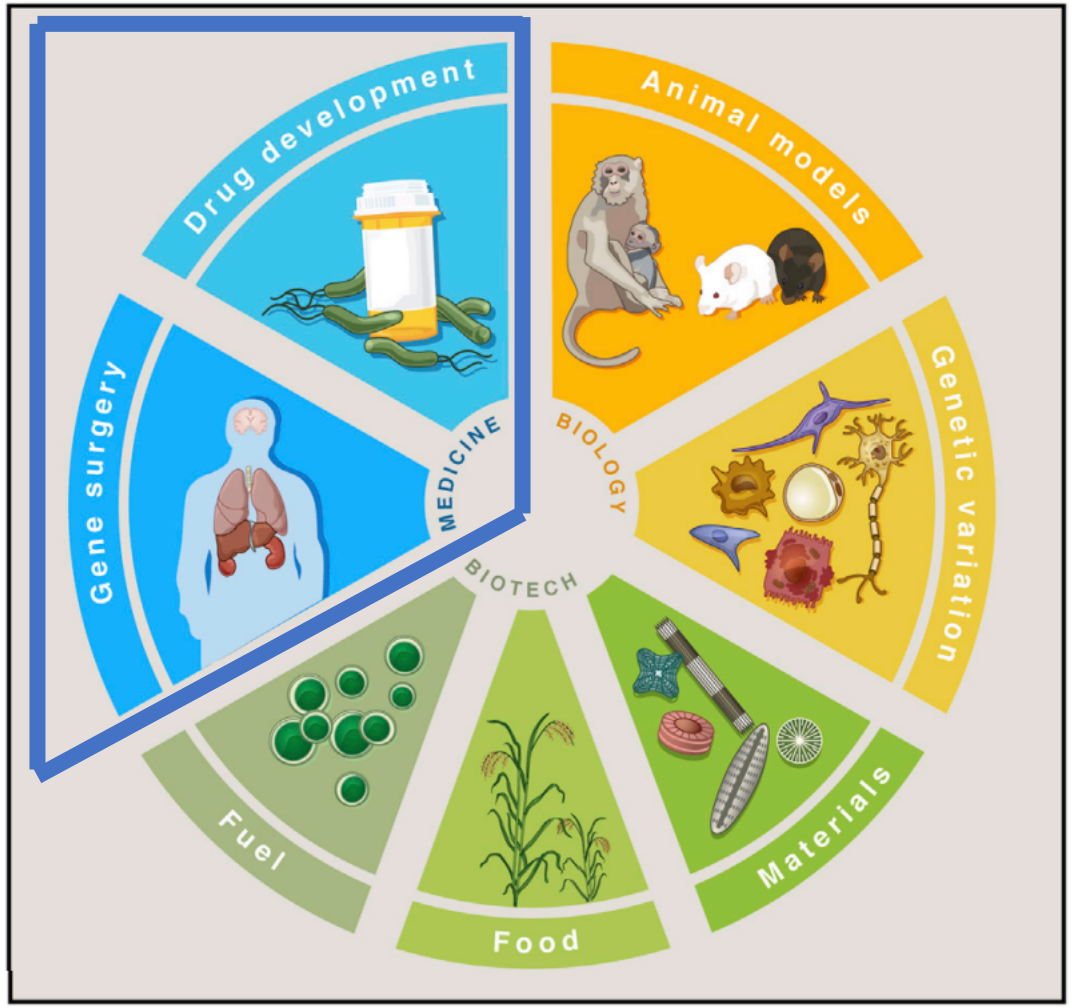
   Targeted nucleases
   - Zinc finger (**ZF**) nucleases based on eukaryotic transcription factors
   - Transcription activator-like effectors (**TALEs**) from *Xanthomonas* bacteria
   - Meganucleases
   - Clustered regularly interspaced short palindromic repeats (**CRISPR**)-**Cas**
     - History
     - Function
3. Genome-editing methods application

Genome engineering

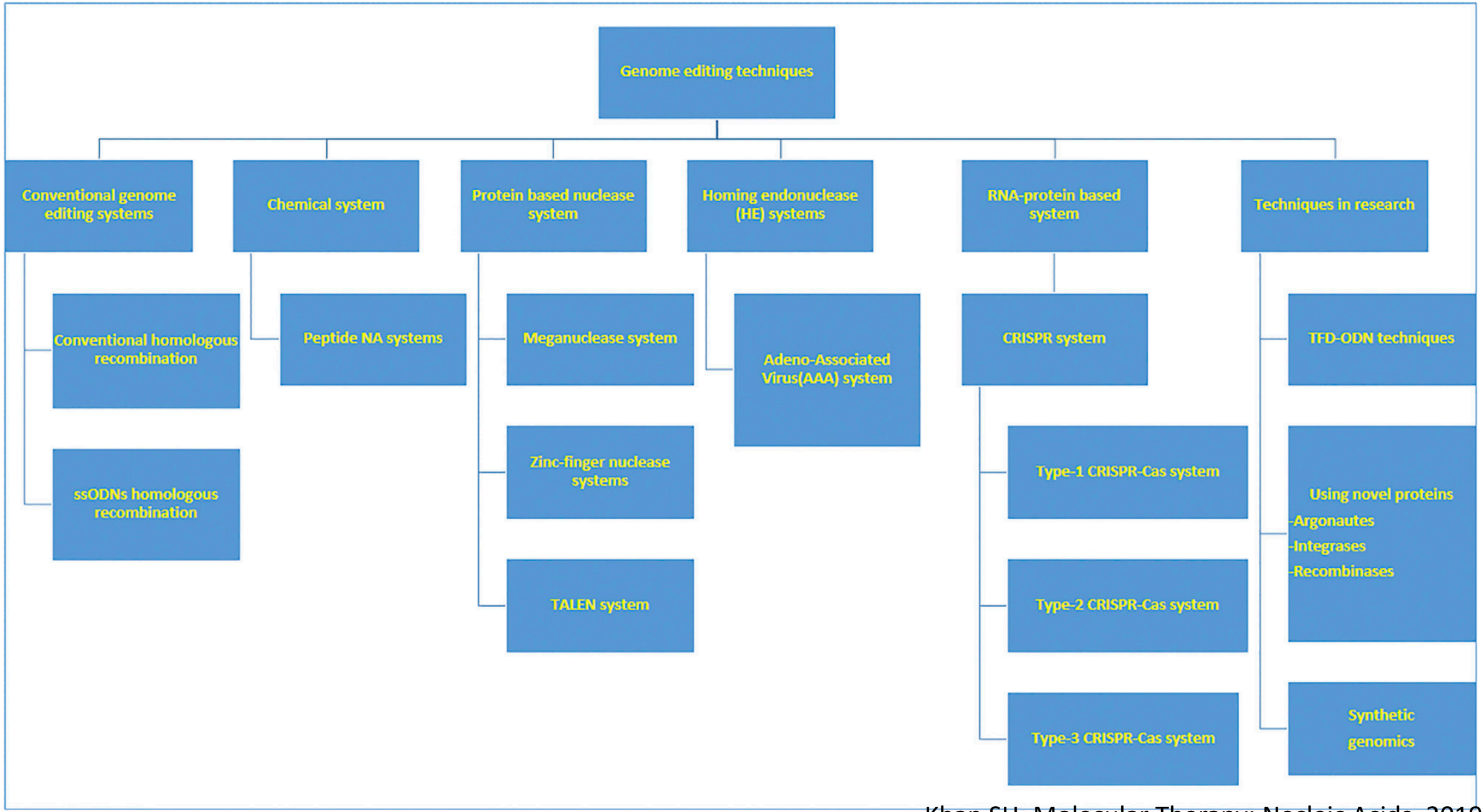


Hsu PD, *et al.*, Cell, 2014

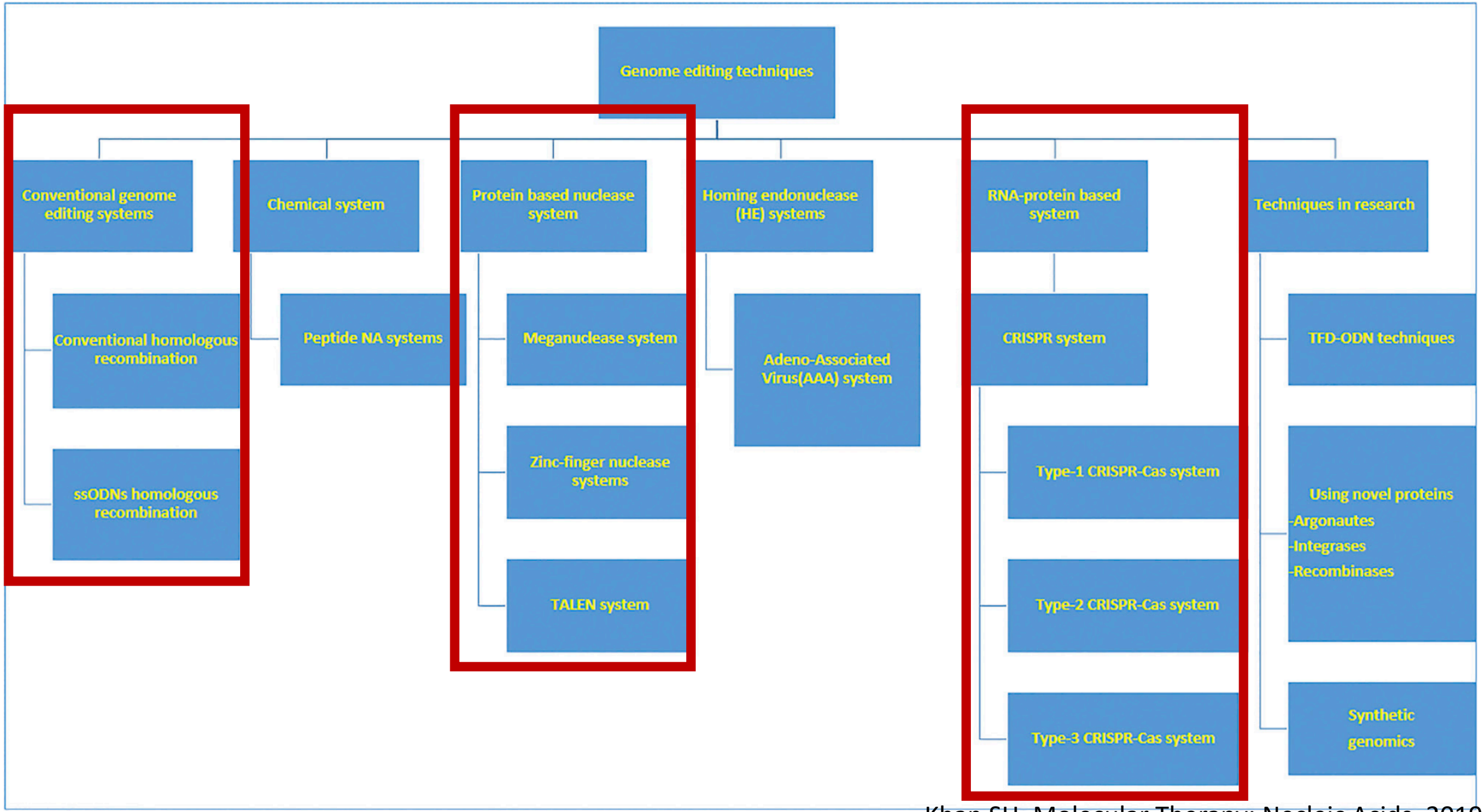
Genome engineering



Hsu PD, *et al.*, Cell, 2014



Khan SH, Molecular Therapy: Necleic Acids, 2019

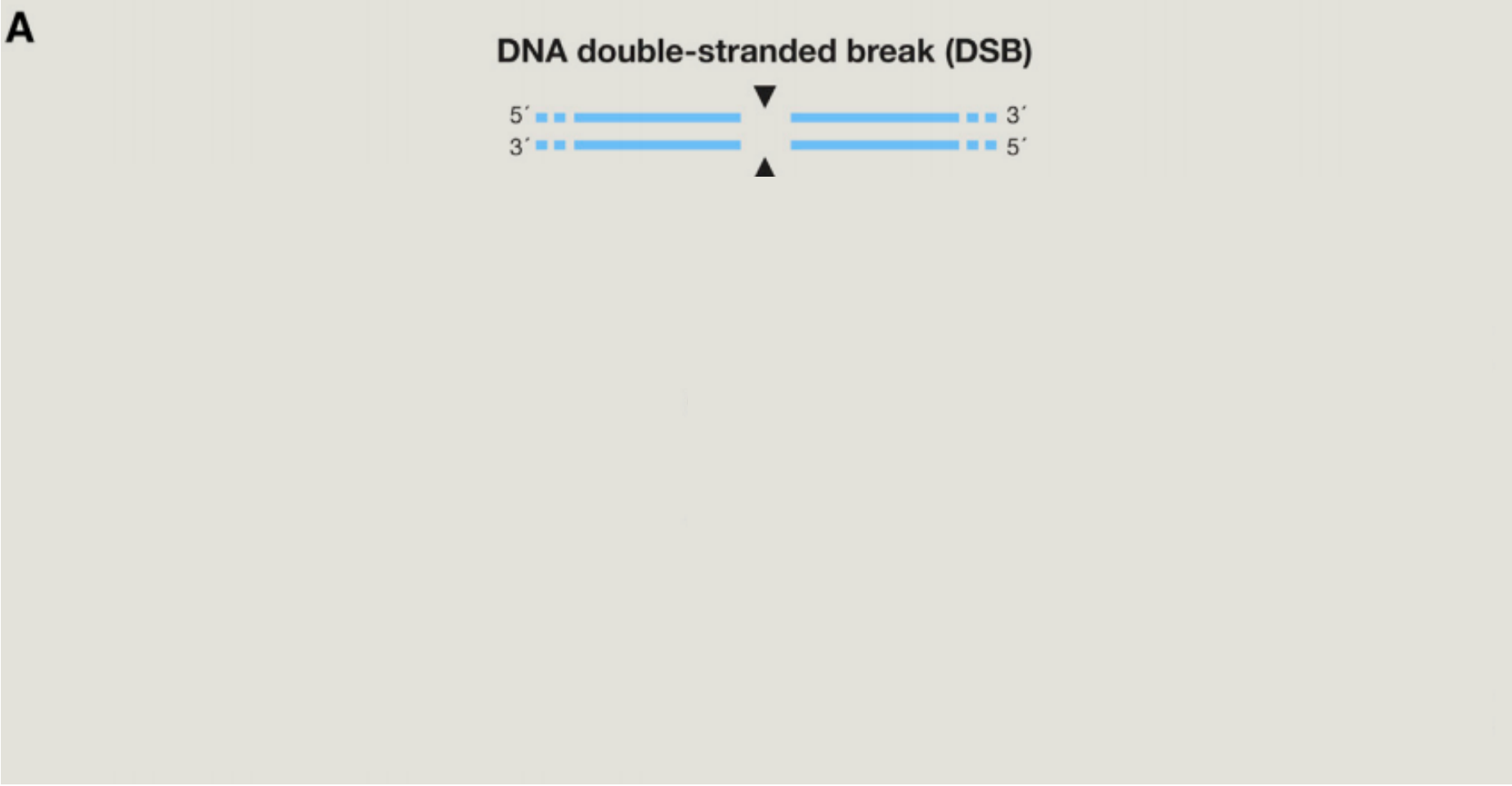


Khan SH, Molecular Therapy: Necleic Acids, 2019

DNA double-stranded break (DSB)

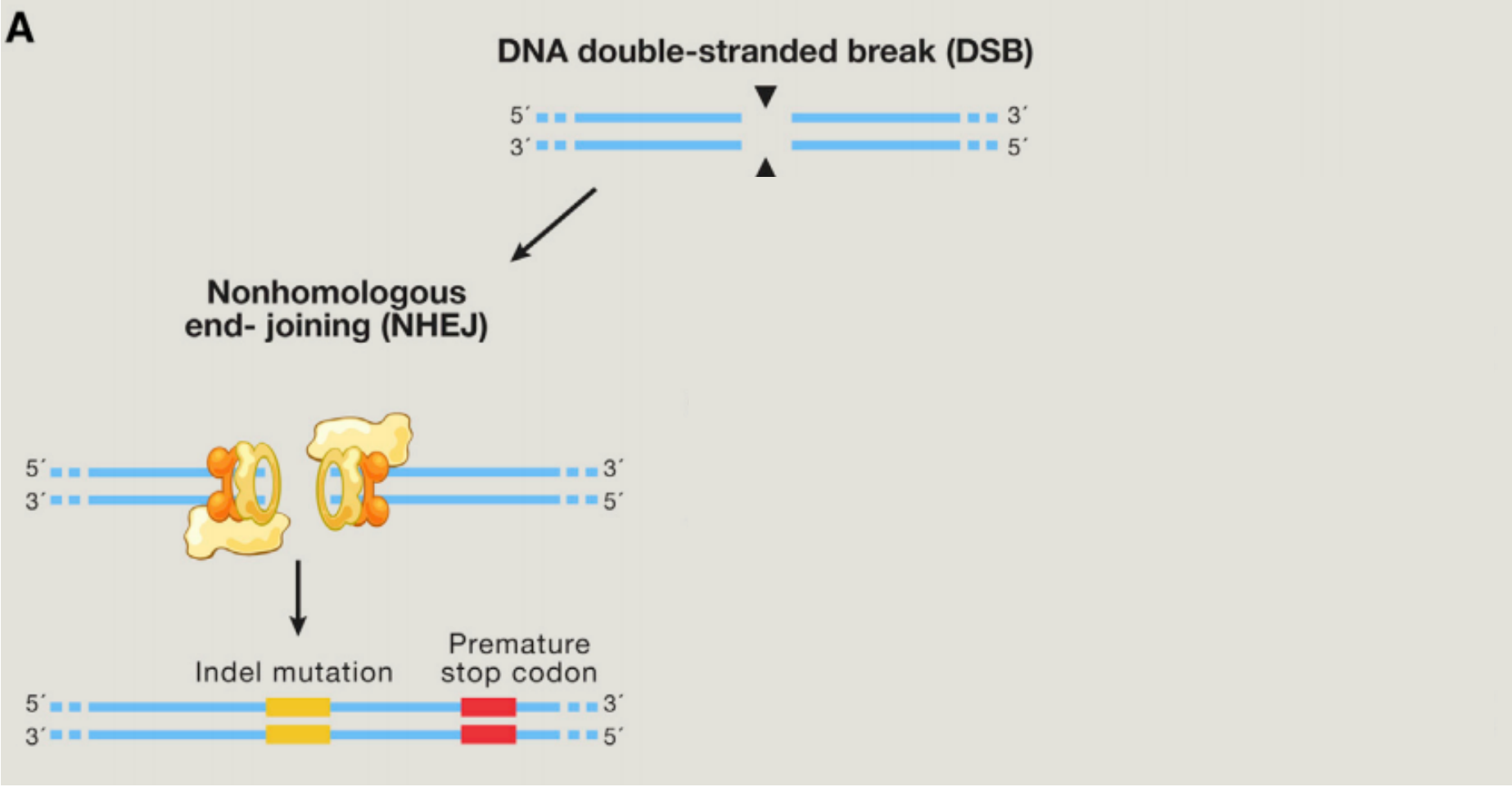
- Known from late 1980s
- Generating knockout and knockin animal models by manipulating germline cells
- Recombination events occure infrequently (1 in 10^6 – 10^9 cells)
- Nonhomologous end-joining (**NHEJ**)
- Homology-directed repair (**HDR**)

DNA double-stranded break (DSB)



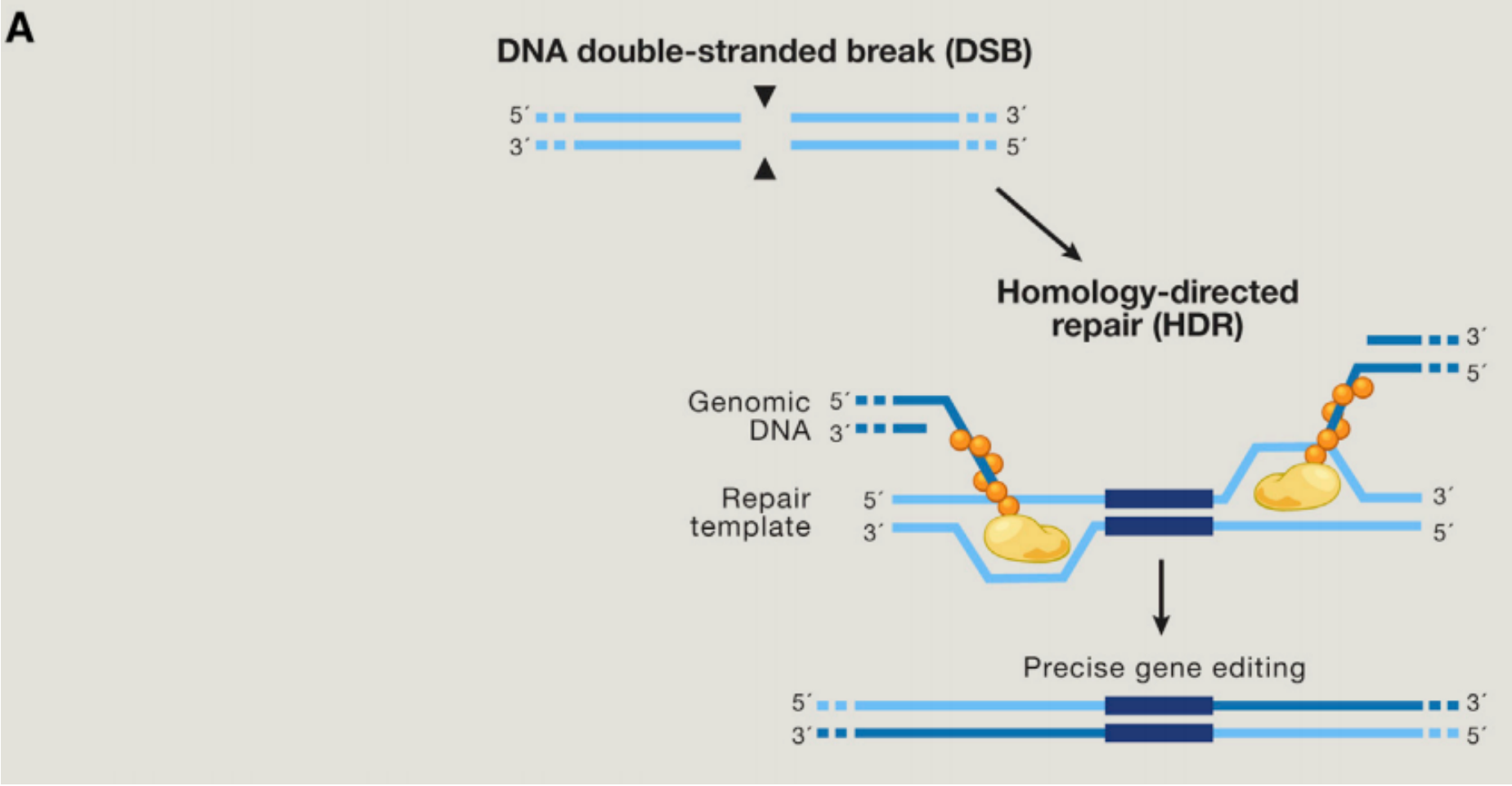
Hsu PD, *et al*., Cell, 2014

DNA double-stranded break (DSB)



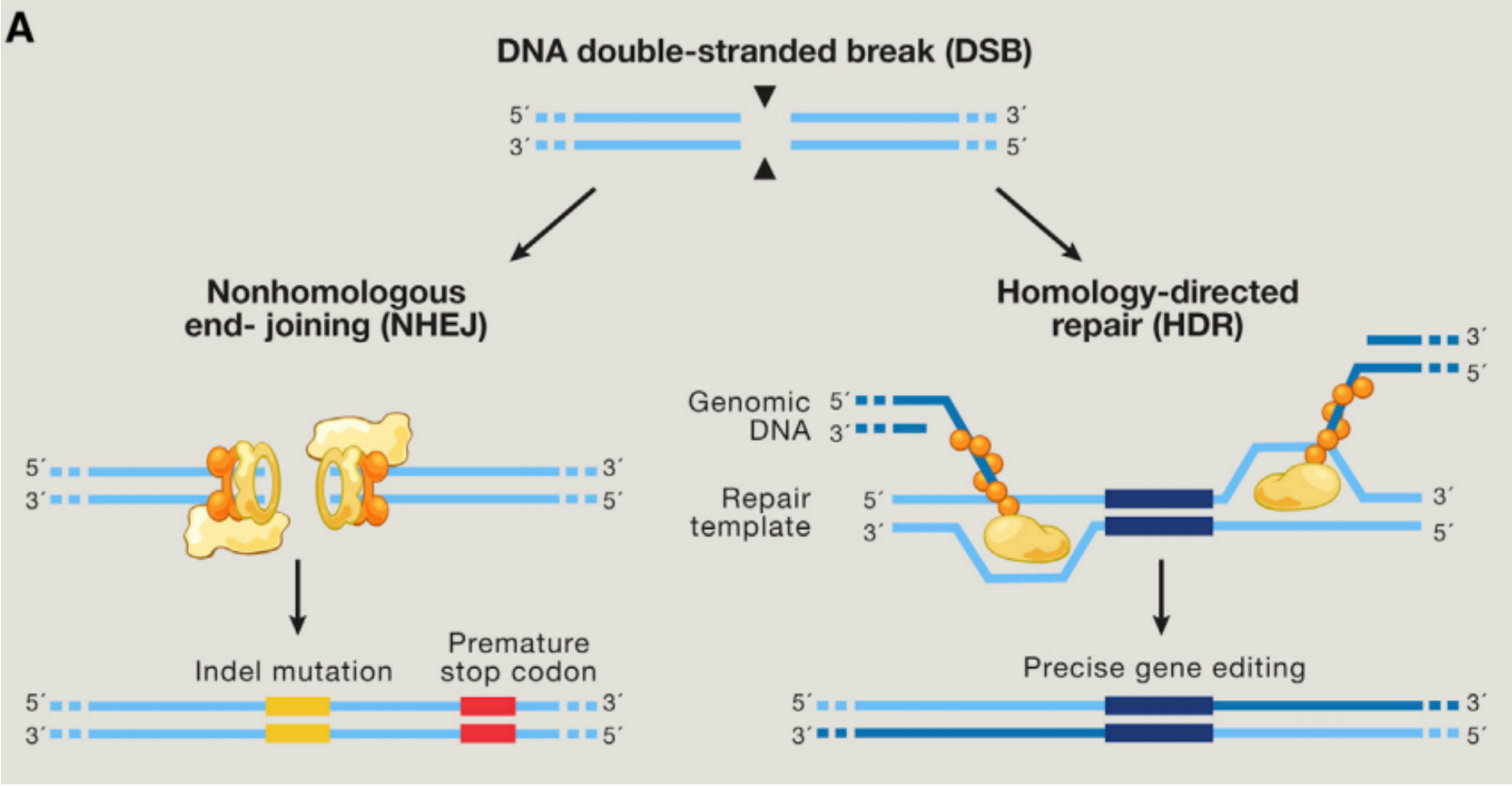
Hsu PD, *et al*., Cell, 2014

DNA double-stranded break (**DSB**)



Hsu PD, *et al.*, Cell, 2014

DNA double-stranded break (**DSB**)



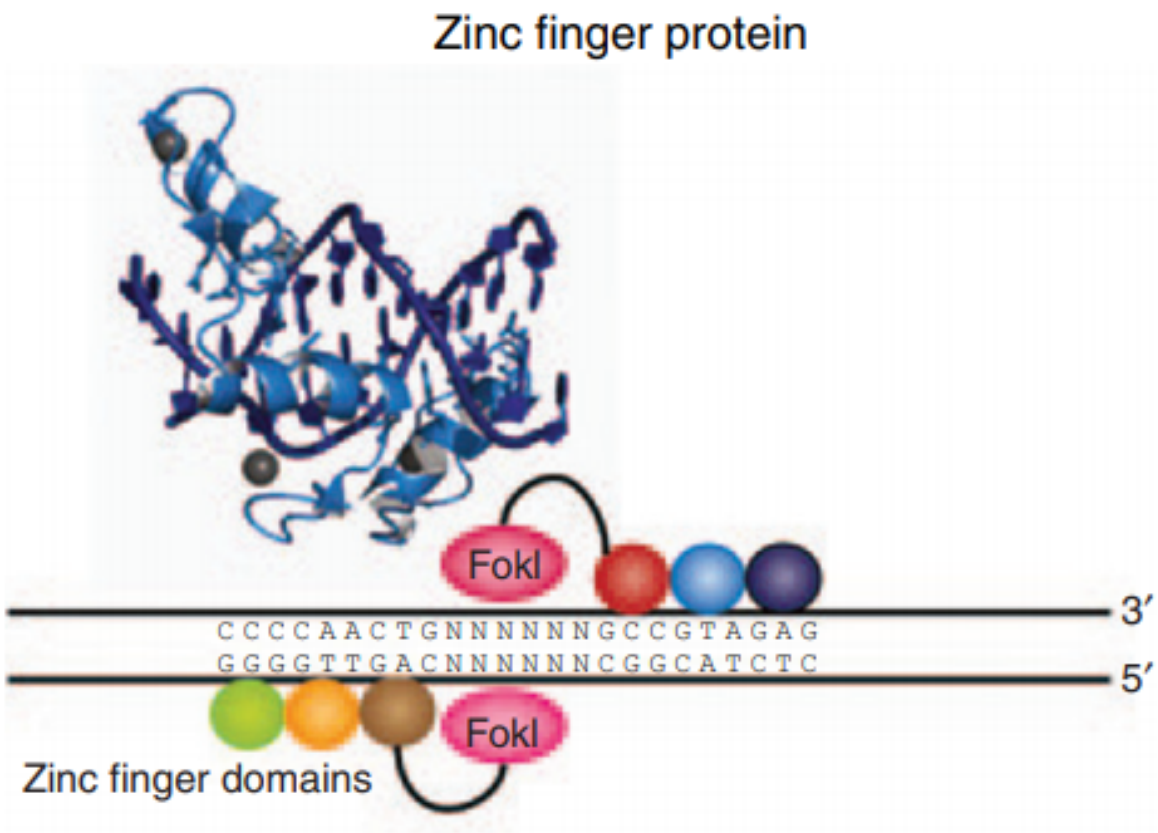
Hsu PD, *et al.*, Cell, 2014

Targeted nucleases – protein based methods

- Zinc finger (**ZF**) nucleases based on eukaryotic transcription factors
- Transcription activator-like effectors (**TALEs**) from *Xanthomonas* bacteria
- Meganucleases
- Clustered regularly interspaced short palindromic repeats (**CRISPR**)-**Cas**

- In the last 10 years more than 4 000 papers with the keywords gene-editing or genome-editing techniques were published
  - DSB – 100 papers
  - Chemical methods - 252 papers
  - ZF – 890 papers
  - TALEs – 1 136 papers
  - Meganucleases – 83 papers
  - CRISPR – 11 421 papers

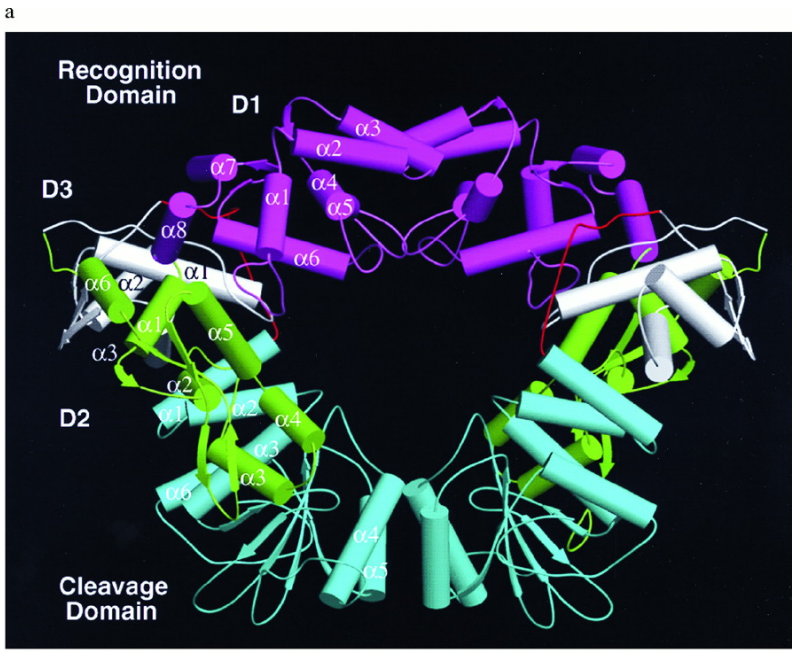
Zinc finger (ZF) nucleases

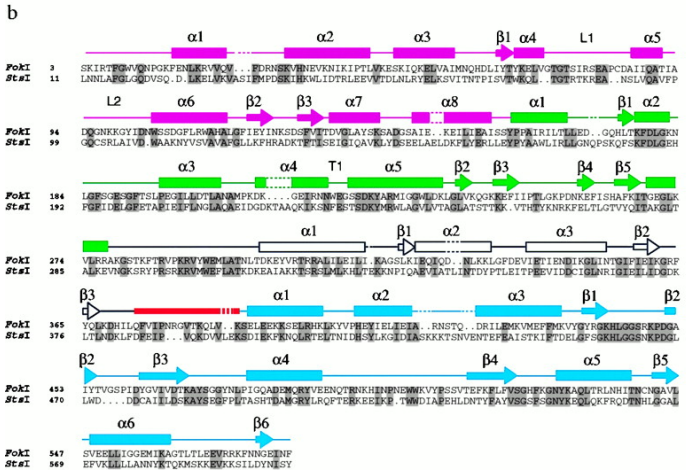


- *Fok*I restriction endonuclease

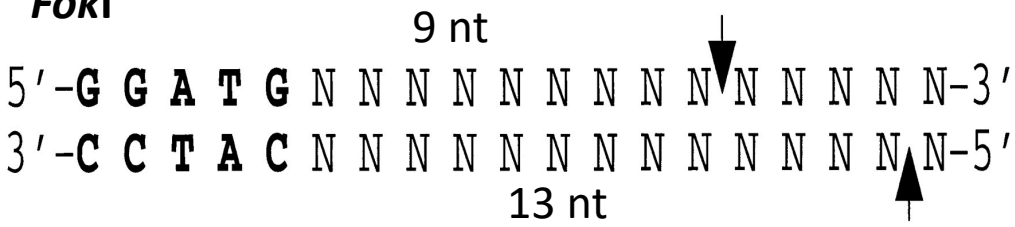
Maede ML & Gersbach CA, Molecular Therapy, 2016

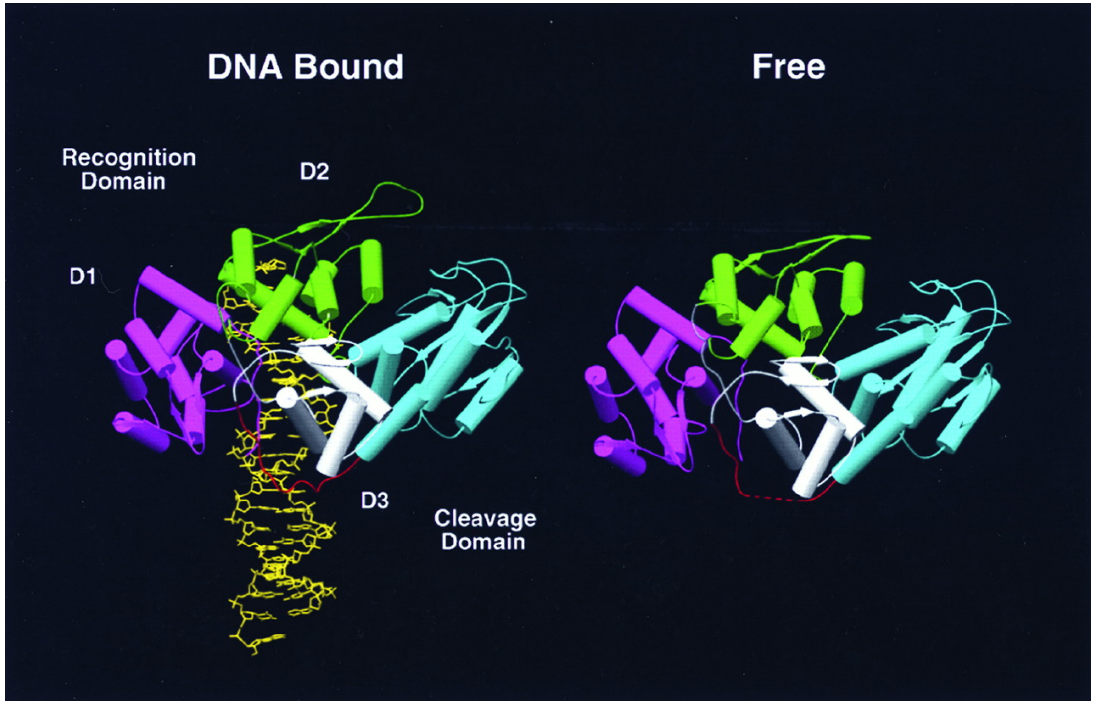
Zinc finger (ZF) nucleases





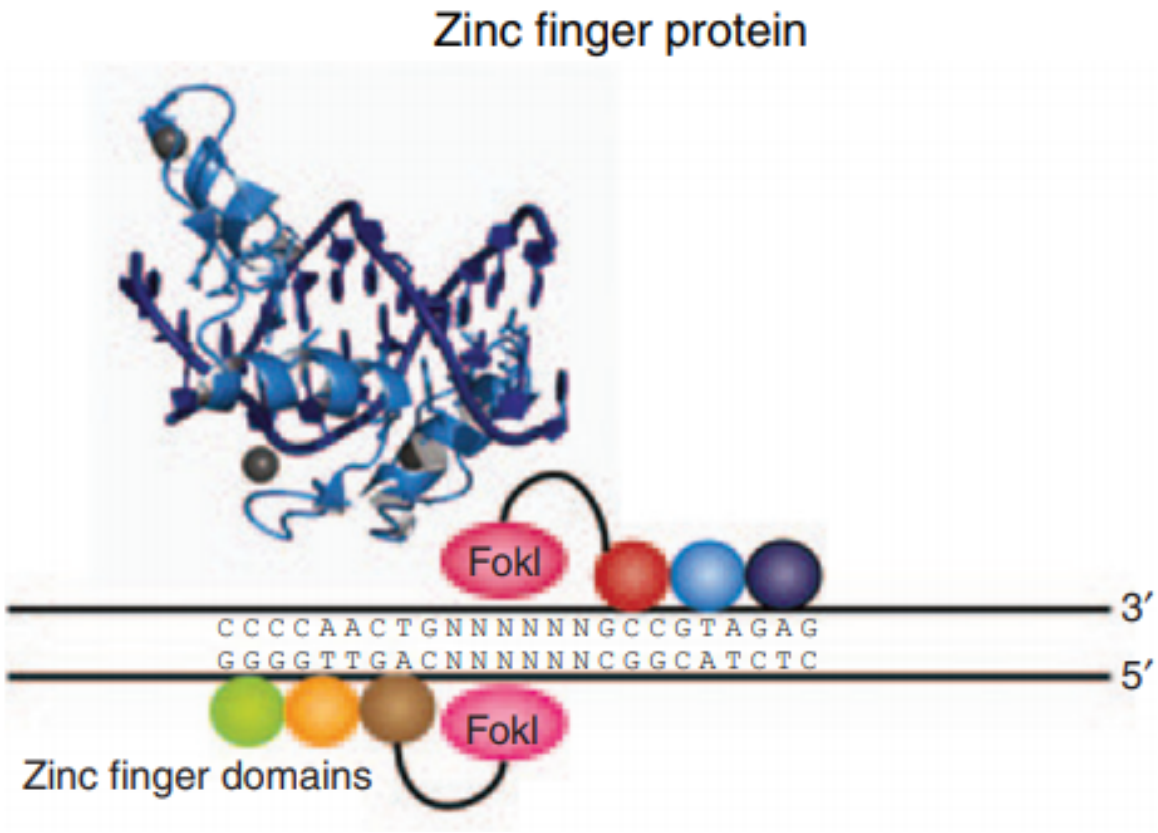
***Fok*I**





Wah DA, *et al.*, PNAS, 1998

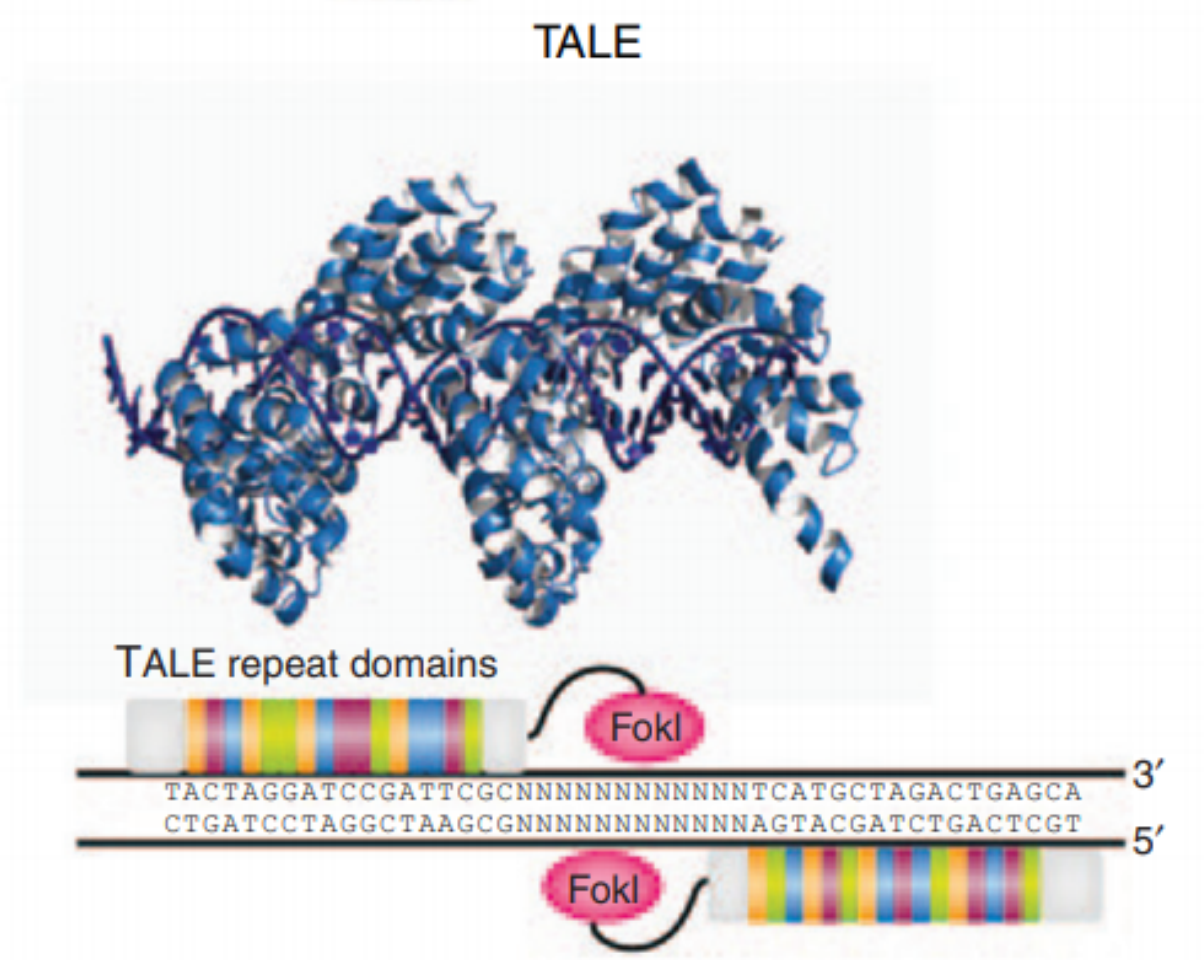
Zinc finger (ZF) nucleases



- *Fok*I restriction endonuclease
- Replacement *Fok*I DNA-binding domain with ZF domain
- ZF finger recognizes 3 bp

Maede ML & Gersbach CA, Molecular Therapy, 2016

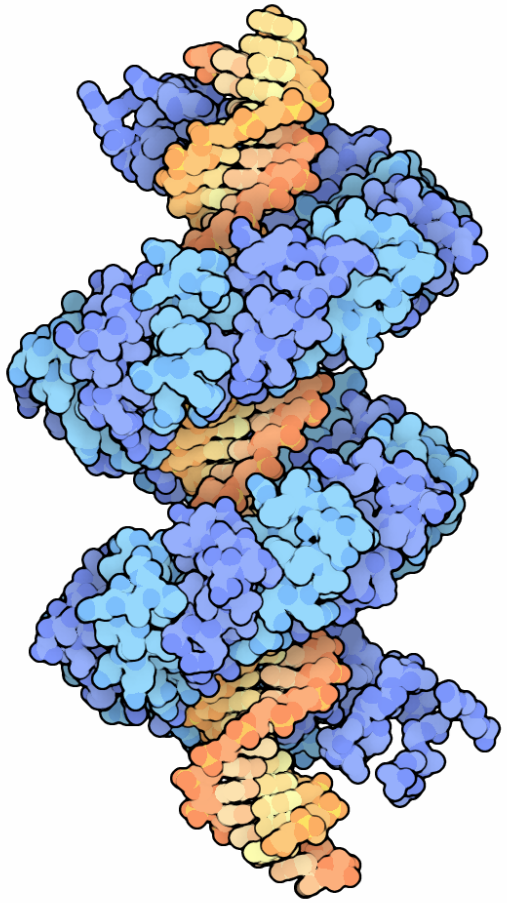
Transcription activator-like effectors (TALEs)



- from the plant pathogen *Xanthomonas*

Maede ML & Gersbach CA, Molecular Therapy, 2016

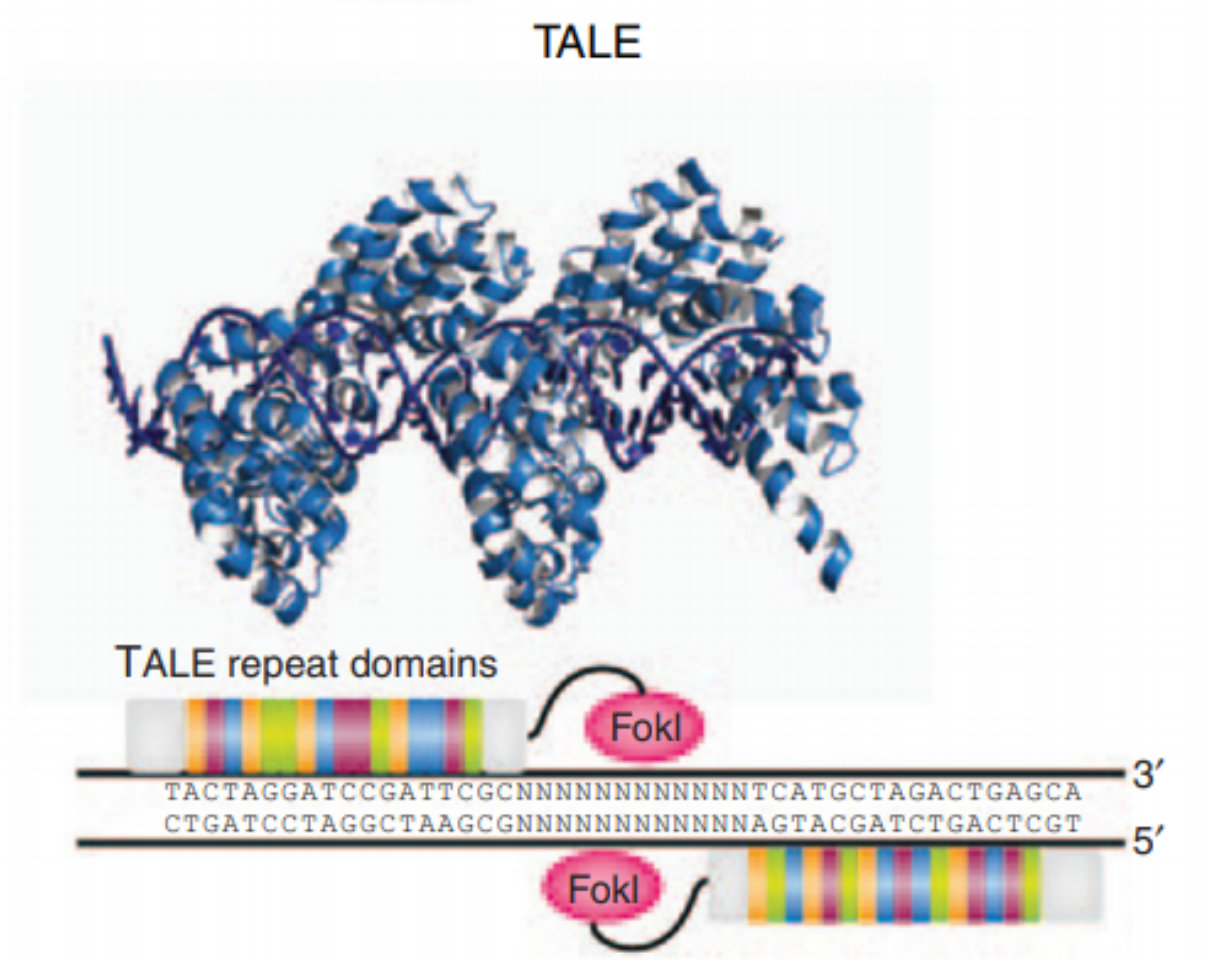
Transcription activator-like effectors (TALEs)



- from the plant pathogen *Xanthomonas*
- TALEs bind promoter sequences in the infected plant
- Activates the expression of plant genes that aid bacterial infection

TAL effector (PDB: 3ugm), spacefill by David Goodsell.
Stripes are repeat domains

Transcription activator-like effectors (TALEs)



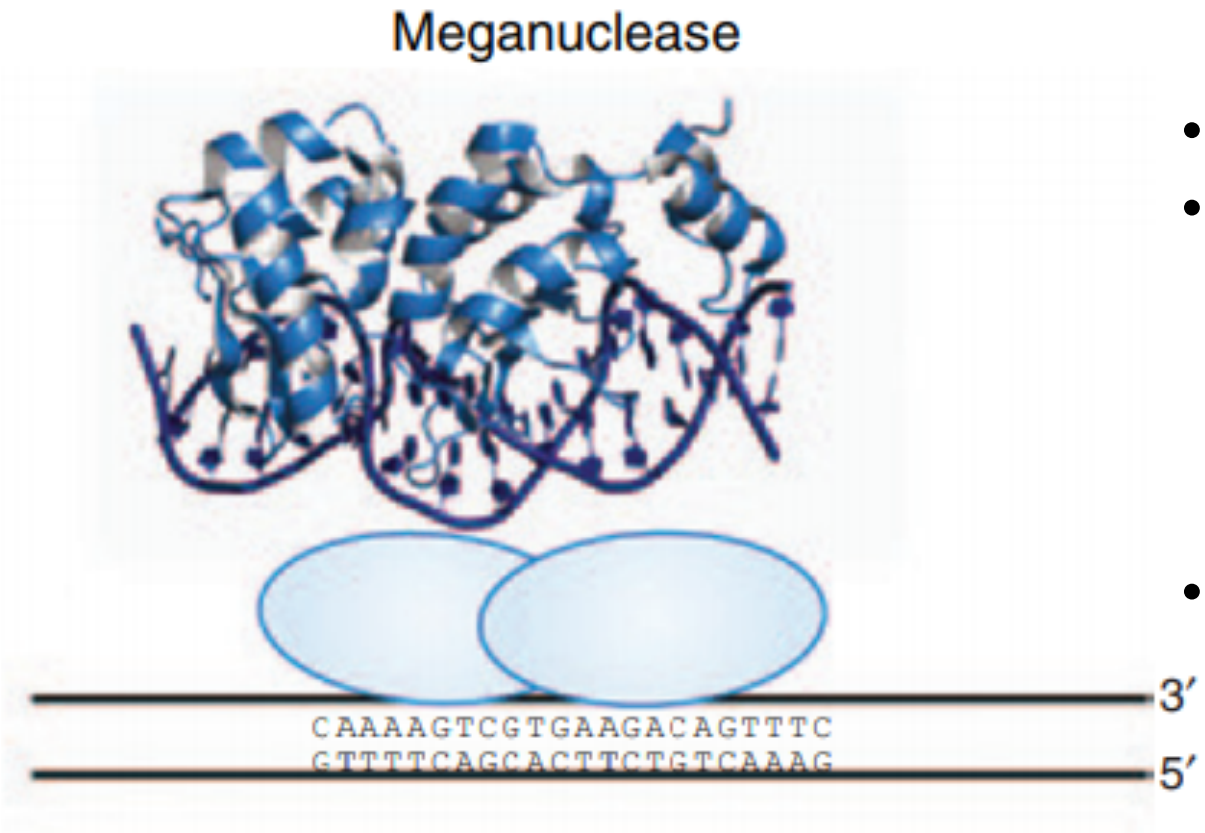
- from the plant pathogen *Xanthomonas*
- DNA-binding domain consists of a variable number of ~34 amino acid repeats
- One repeat recognizes one base pair
- One repeat:

LTPEQVVAIASHDGGKQALETVQRLLPVLCQAHG

Residue	Base
NI	A
HD	C
NG	T, $5^{m}C$
NN	R
NS	N
NK	G
NH	G

Maede ML & Gersbach CA, Molecular Therapy, 2016

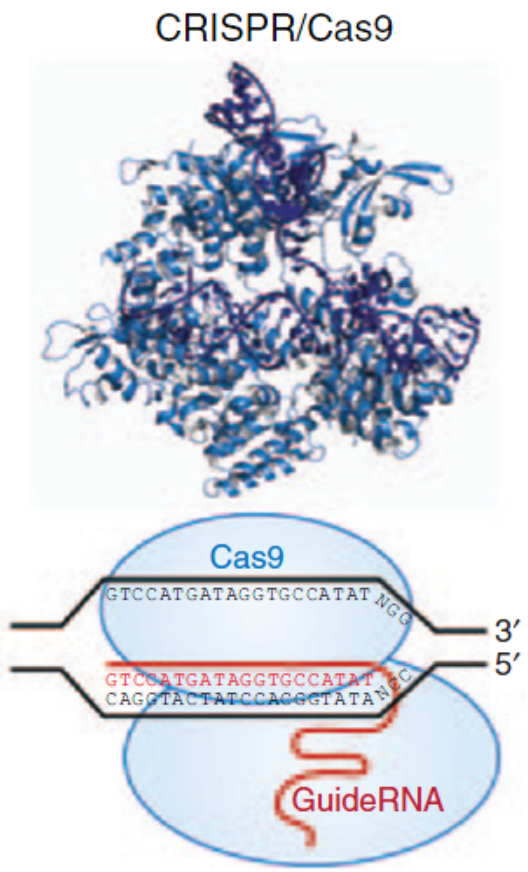
Meganucleases



- Meganuclease technology involves re-engineering the DNA-binding specificity of naturally occurring homing endonucleases
- ”molecular DNA acissors”
- well-characterized and commonly used are
  - I-*Cre*I (mitochondria of the *S. cerevisiae*)
  - I-*Sce*I (chloroplasts of the *Chlamydomonas reinhardtii*)
  - I-*Dmo*I (archaebacterium *Desulfurococcus mobilis*)
- homing endonucleases are difficult to separate, and the relative difficulty of engineering proteins

Maede ML & Gersbach CA, Molecular Therapy, 2016

Clustered regularly interspaced short palindromic repeats (**CRISPR**)-**Cas**



- derived from an adaptive immune system that evolved in bacteria
- Type II CRISPR system: CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA), Cas9 protein (from *Streptococcus pyogenes*)
- Guide RNA (gRNA) = crRNA + tracrRNA
- protospacer-adjacent motif (PAM) – DNA sequence located on 3’ site of tergeted sequence
  - 5’-NGG-3’ is necessary for binding and cleavage of DNA by the Cas9

Maede ML & Gersbach CA, Molecular Therapy, 2016

Comparison of genome-editing methods

Table 1. Biotechnology Differences among Prototype Genome-Editing Techniques

Serial No.	Parameter	ZFN	TALEN	CRISPER/Cas
1	design simplicity	moderate (ZFNs need customized protein for every DNA sequence)	slightly complex (identical repeats are multiple, which creates technical issues of engineering and delivery into cells)	simpler (available versions for crRNA can be easily designed)
2	engineering feasibility	low	higher	highest
3	multiplex genome editing	few models	few models	high-yield multiplexing available (no need for obtaining embryonic stem cells)
4	large-scale library preparation	not much progress (need individual gene tailoring)	not much progress (need individual gene tailoring)	progress demonstrated (CRISPR only requires plasmid containing small oligonucleotides)
5	specificity	low	higher	highest
6	efficiency	normal[a]	normal[b]	high
7	cost	low	high	low

[a]Some new versions are more efficient[24,48] but CRISPR science is evolving more.
[b]Cpf1 protein addition will probably improve cell delivery methods.[51,52]

Based on: Khan SH, Molecular Therapy: Necleic Acids, 2019

Comparison of genome-editing methods

Table 2. Side Effect Profiles for Genome-Editing Methods

Serial No.	Parameter	ZFN	TALEN	CRISPER/Cas
1	off-target effect incidence	–	–	–
a	homologous recombination rate frequency	+	+	+
b	non-homologous end joining (NHEJ) mutation rates	+	+	++ (only with earlier versions)
c	immune reaction susceptibility	less	less	more
d	RNA-guided endonuclease (RGEN)-induced off-target mutatagenesis	–	–	++
2	cytotoxicity chances	++	+	+

Based on: Khan SH, Molecular Therapy: Necleic Acids, 2019

Comparison of genome-editing methods

Table 3. Clinical and Research Applications across Important Genome-Editing Techniques

Serial No.	Parameter	ZFN	TALEN	CRISPER/Cas
1	diagnostic utility	+	+	+++
2	clinical trial use	++	+	+++
3	utility as epigenetic marker	++	+++	++++
4	making gene-knockout models for research	no	no	yes (CRISPRi)
5	capacity for modification of mitochondrial DNA	no	no	probable
6	genetic editing in human babies	no	no	yes
7	RNA editing	no	no	yes

Based on: Khan SH, Molecular Therapy: Necleic Acids, 2019

Com

Table 3. C
Editing Te

Serial No.
1
2
3
4
5
6
7

A bacterial cytidine deaminase toxin enables CRISPR-free mitochondrial base editing

hods

https://doi.org/10.1038/s41586-020-2477-4

Received: 25 January 2020

Accepted: 26 May 2020

Published online: 08 July 2020

Check for updates

Beverly Y. Mok[1,2,3,11], Marcos H. de Moraes[4,11], Jun Zeng[4], Dustin E. Bosch[4,5], Anna V. Kotrys[8,9,10], Aditya Raguram[1,2,3], FoSheng Hsu[4], Matthew C. Radey[4], S. Brook Peterson[4], Vamsi K. Mootha[8,9], Joseph D. Mougous[4,6,7]✉ & David R. Liu[1,2,3]✉

Bacterial toxins represent a vast reservoir of biochemical diversity that can be repurposed for biomedical applications. Such proteins include a group of predicted interbacterial toxins of the deaminase superfamily, members of which have found application in gene-editing techniques[1,2]. Because previously described cytidine deaminases operate on single-stranded nucleic acids[3], their use in base editing requires the unwinding of double-stranded DNA (dsDNA)—for example by a CRISPR–Cas9 system. Base editing within mitochondrial DNA (mtDNA), however, has thus far been hindered by challenges associated with the delivery of guide RNA into the mitochondria[4]. As a consequence, manipulation of mtDNA to date has been limited to the targeted destruction of the mitochondrial genome by designer nucleases[9,10].Here we describe an interbacterial toxin, which we name DddA, that catalyses the deamination of cytidines within dsDNA. We engineered split-DddA halves that are non-toxic and inactive until brought together on target DNA by adjacently bound programmable DNA-binding proteins. Fusions of the split-DddA halves, transcription activator-like effector array proteins, and a uracil glycosylase inhibitor resulted in RNA-free DddA-derived cytosine base editors (DdCBEs) that catalyse C•G-to-T•A conversions in human mtDNA with high target specificity and product purity. We used DdCBEs to model a disease-associated mtDNA mutation in human cells, resulting in changes in respiration rates and oxidative phosphorylation. CRISPR-free DdCBEs enable the precise manipulation of mtDNA, rather than the elimination of mtDNA copies that results from its cleavage by targeted nucleases, with broad implications for the study and potential treatment of mitochondrial disorders.

Based on: Khan SH, Molecular Therapy: Necleic Acids, 2019

Comparison of genome-editing methods

Table 3. Clinical and Research Applications across Important Genome-Editing Techniques

Serial No.	Parameter	ZFN	TALEN	CRISPER/Cas
1	diagnostic utility	+	+	+++
2	clinical trial use	++	+	+++
3	utility as epigenetic marker	++	+++	++++
4	making gene-knockout models for research	no	no	yes (CRISPRi)
5	capacity for modification of mitochondrial DNA	no	no	probable
6	genetic editing in human babies	no	no	yes
7	RNA editing	no	no	yes

Based on: Khan SH, Molecular Therapy: Necleic Acids, 2019

Clustered regularly interspaced short palindromic repeats (**CRISPR**)-**Cas**

- Adaptive immune system
  - 50% bacteria
  - 90% archea
- 93 *cas* genes, grouped into 35 families
- The best explored method
  - In the last 10 years more than 4 000 papers with the keywords gene-editing or genome-editing techniques were published
    - DSB – 100 papers
    - Chemical methods - 252 papers
    - Meganucleases – 83 papers
    - ZF – 890 papers
    - TALEN – 1 136 papers
    - **CRISPR – 11 421 papers**

According to Khan SH, Molecular Therapy: Necleic Acids, 2019

The Nobel Prize in Chemistry 2020

© Nobel Media. Ill. Niklas Elmehed.

Emmanuelle Charpentier

Prize share: 1/2

© Nobel Media. Ill. Niklas Elmehed.

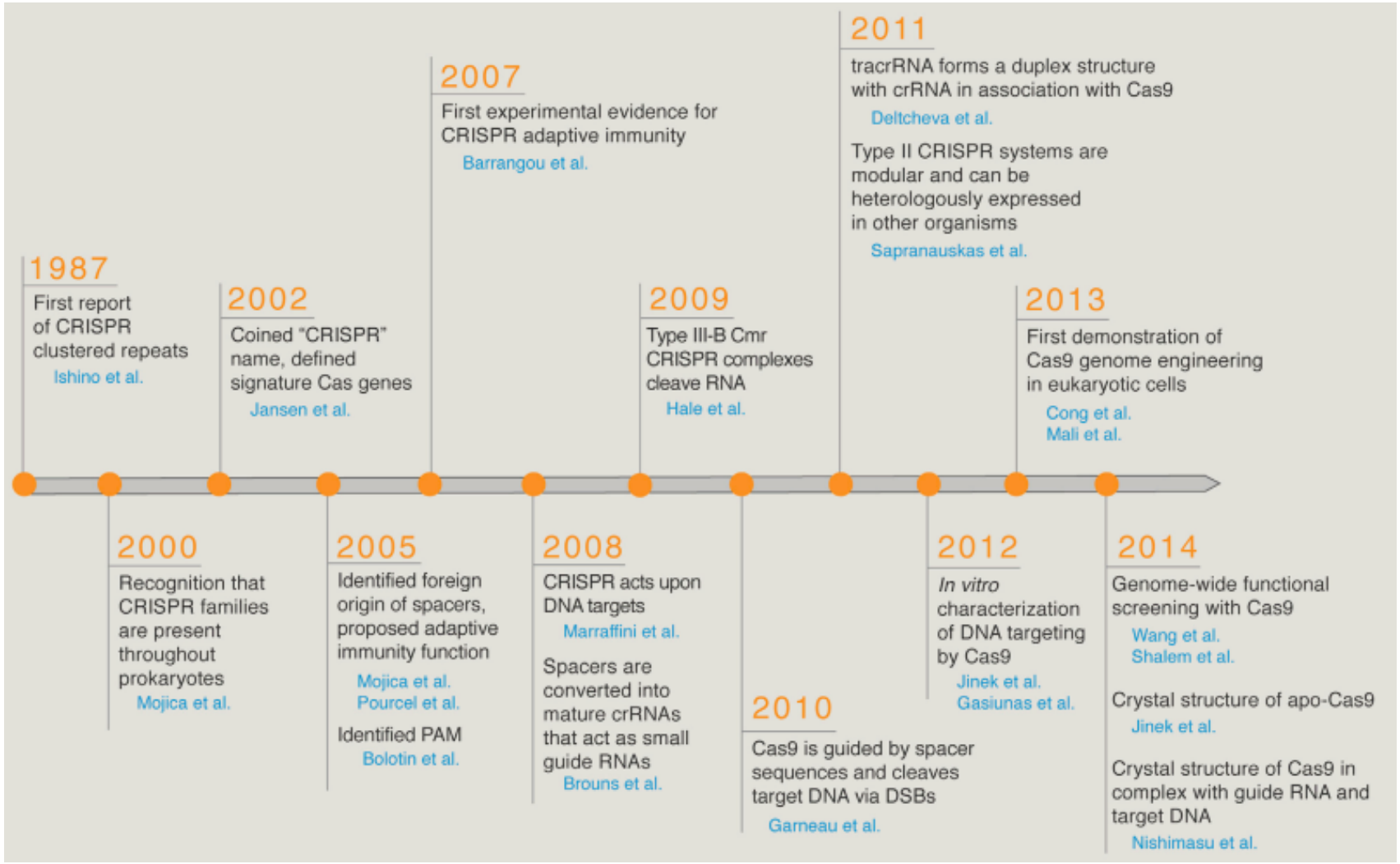
Jennifer A. Doudna

Prize share: 1/2

"for the development of a method for genome editing."

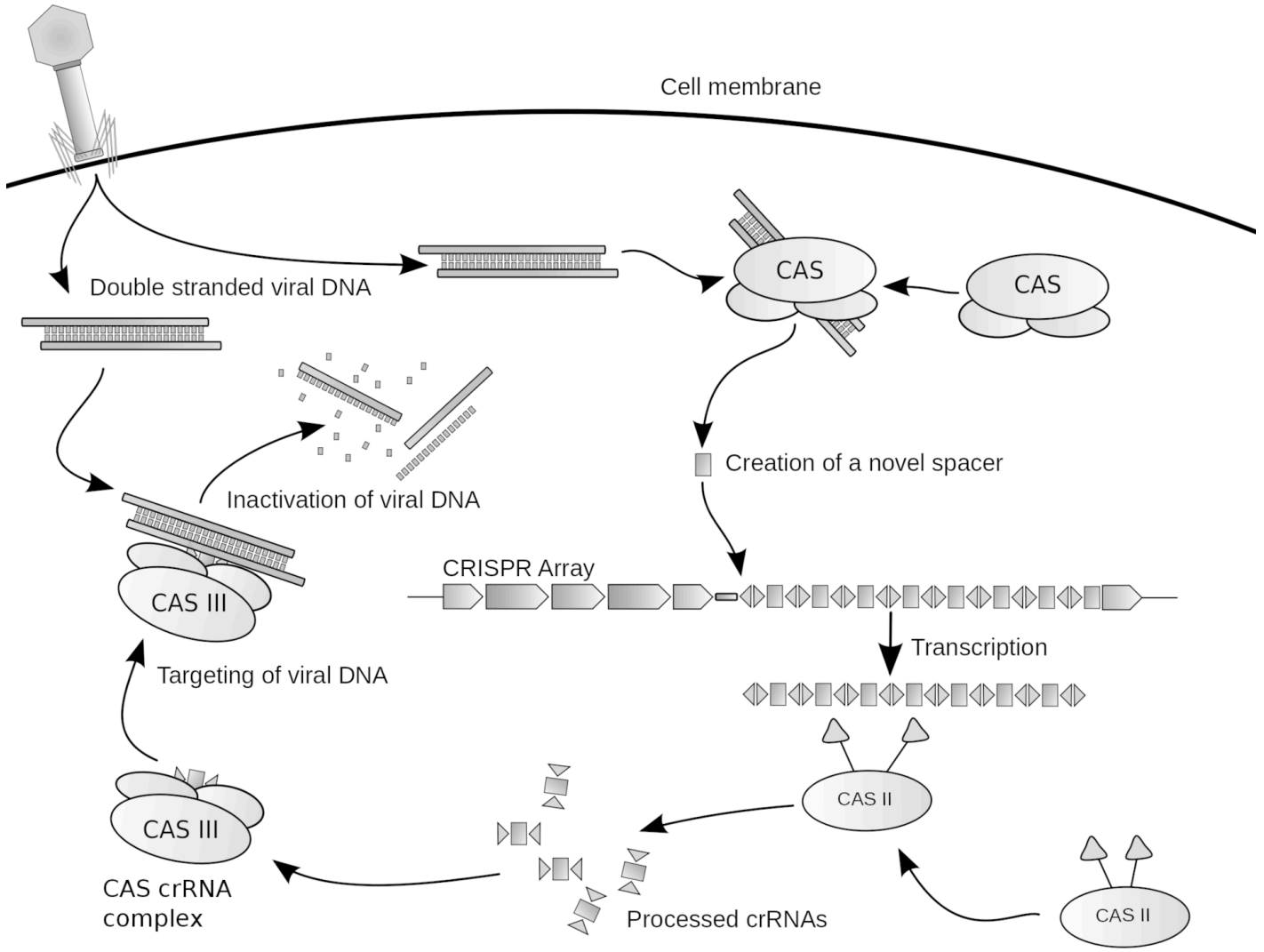
https://www.nobelprize.org/prizes/chemistry/2020/summary/

Clustered regularly interspaced short palindromic repeats (**CRISPR**)-**Cas** - history



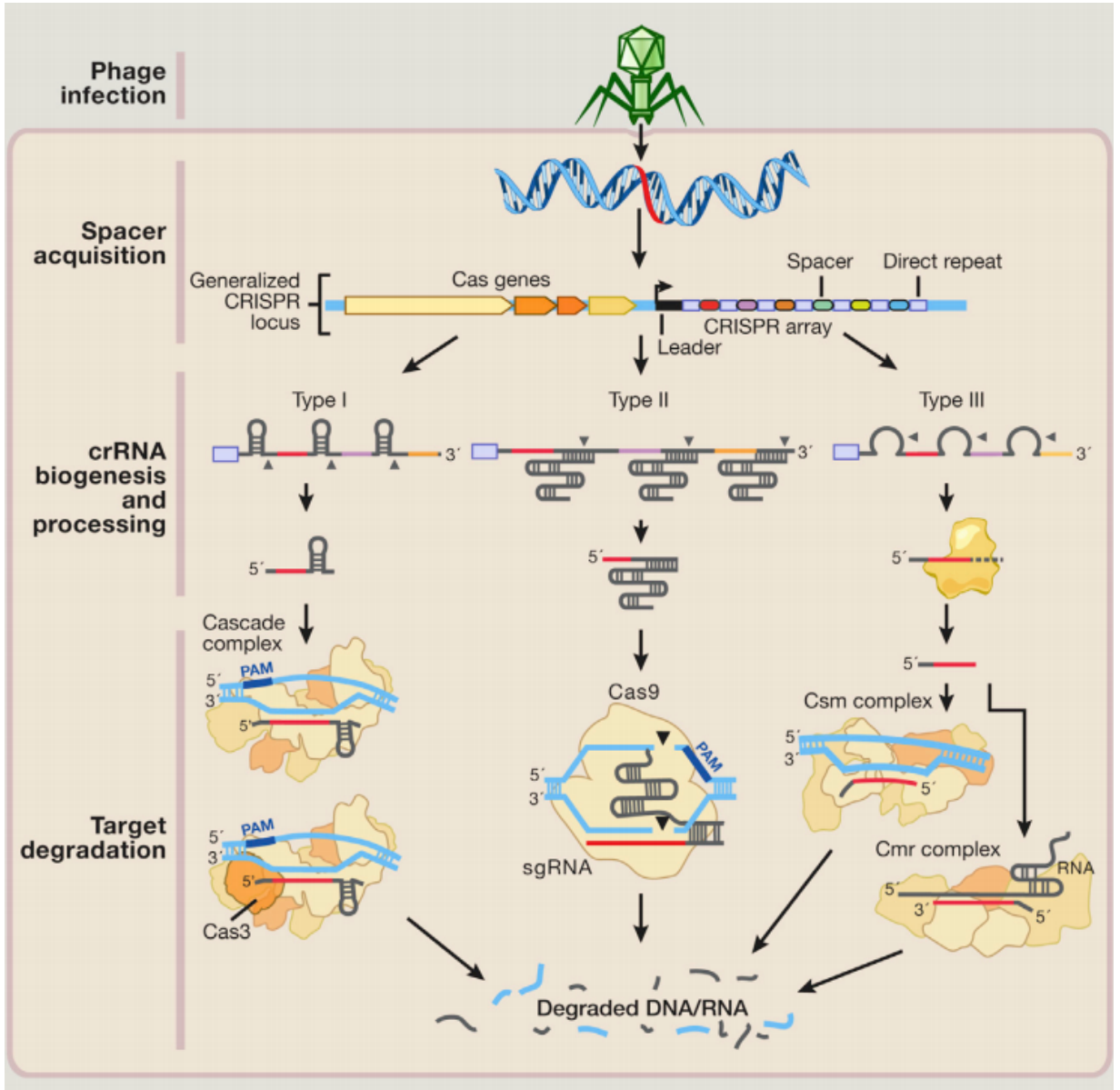
Hsu PD, *et al.*, Cell, 2014

Clustered regularly interspaced short palindromic repeats (**CRISPR**)-**Cas** - function



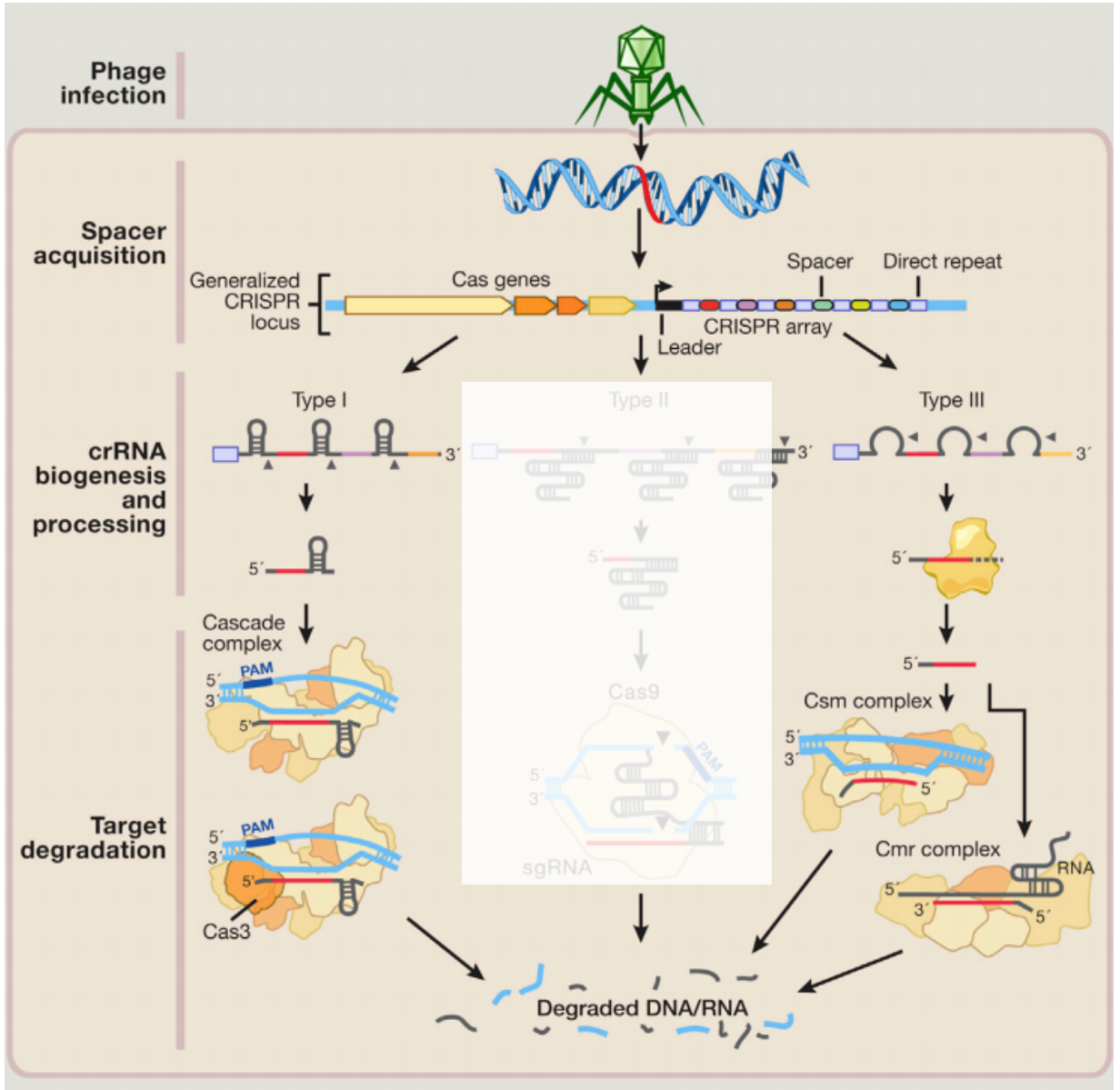
https://en.wikipedia.org/wiki/CRISPR#/media/File:Crispr.png

Clustered regularly interspaced short palindromic repeats (**CRISPR**)-**Cas** - function



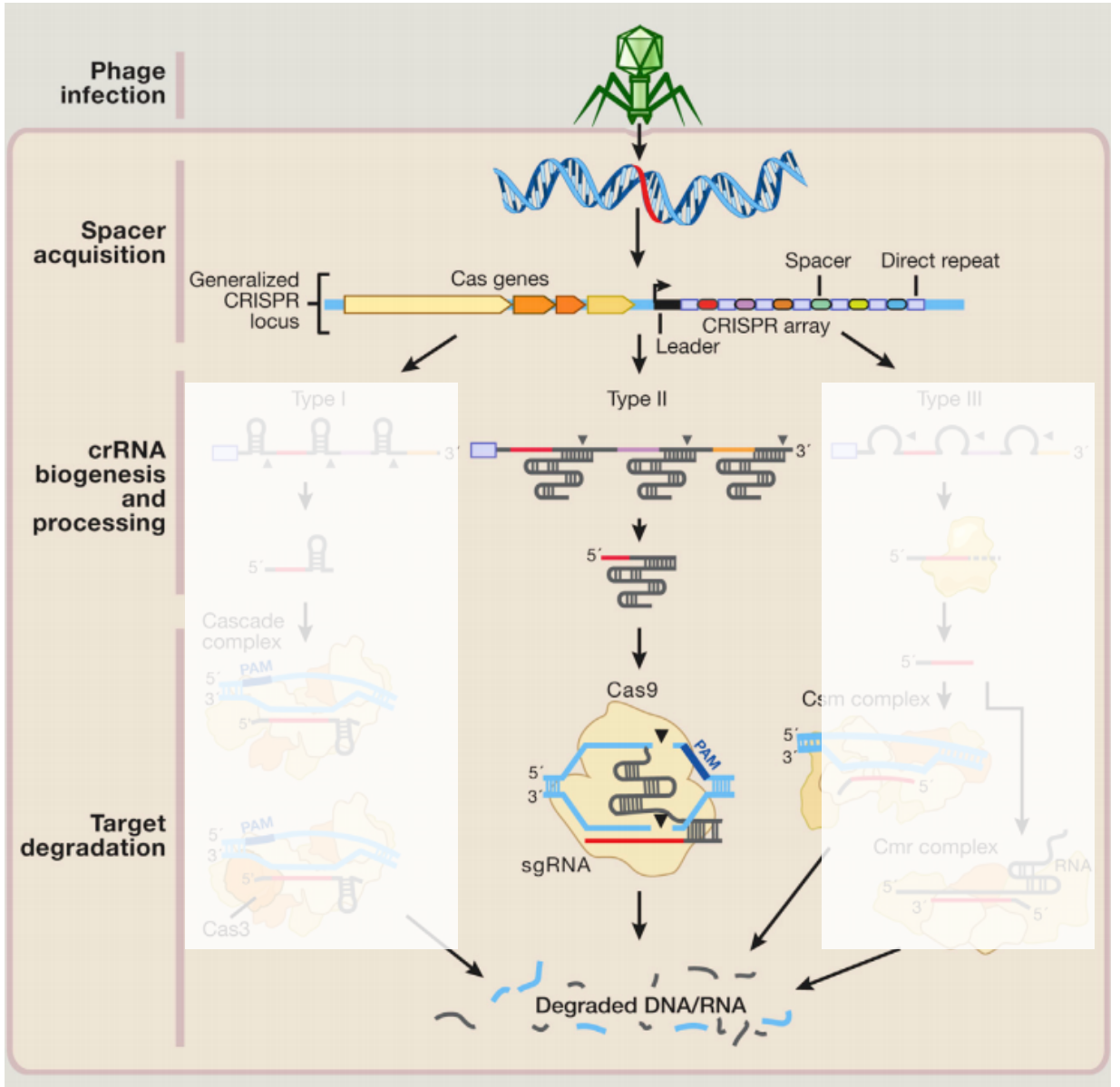
Hsu PD, *et al.*, Cell, 2014

Clustered regularly interspaced short palindromic repeats (**CRISPR**)-**Cas** - function



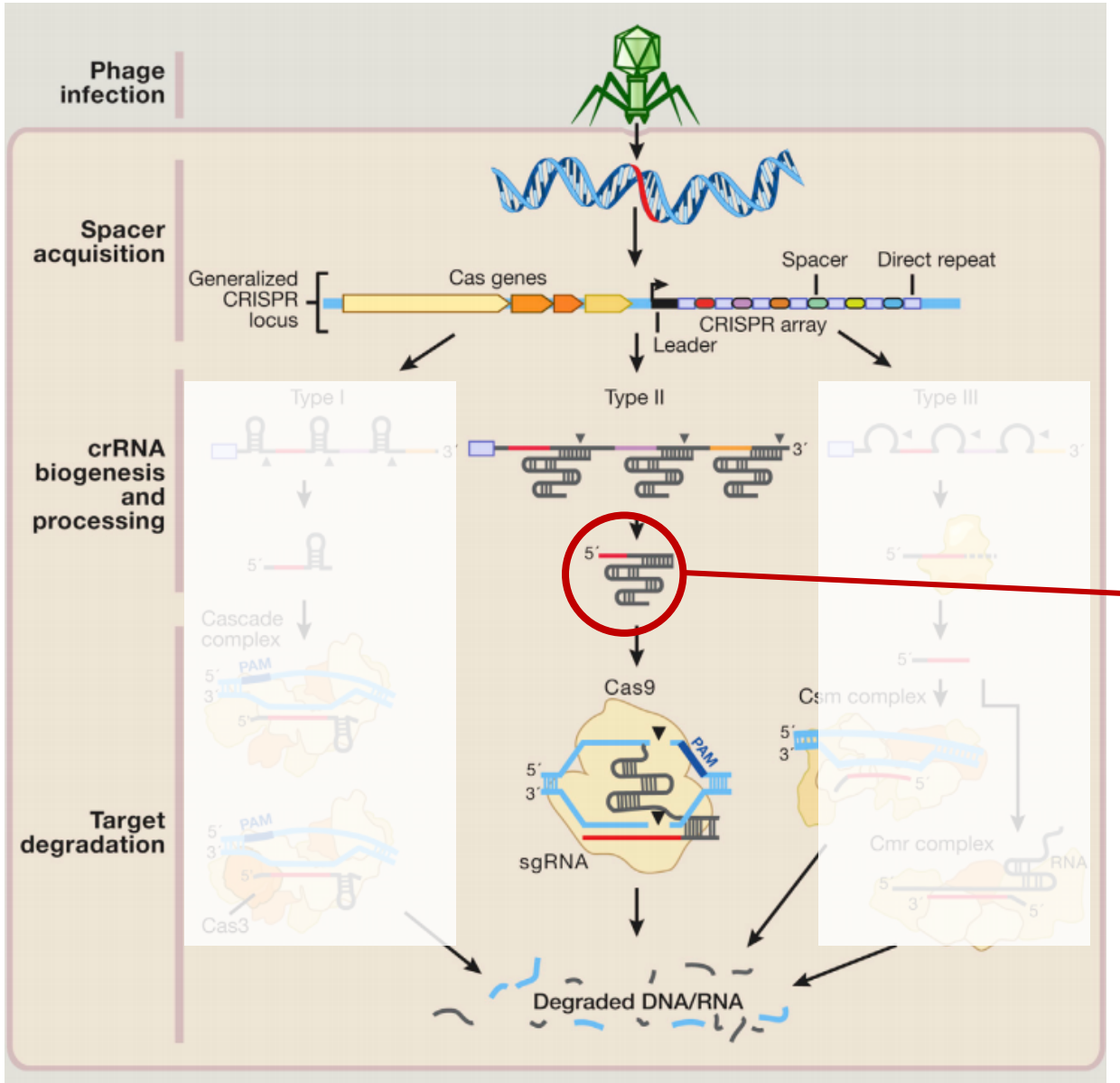
Hsu PD, *et al.*, Cell, 2014

Clustered regularly interspaced short palindromic repeats (**CRISPR**)-**Cas** - function



Hsu PD, *et al.*, Cell, 2014

Clustered regularly interspaced short palindromic repeats (**CRISPR**)-**Cas** - function



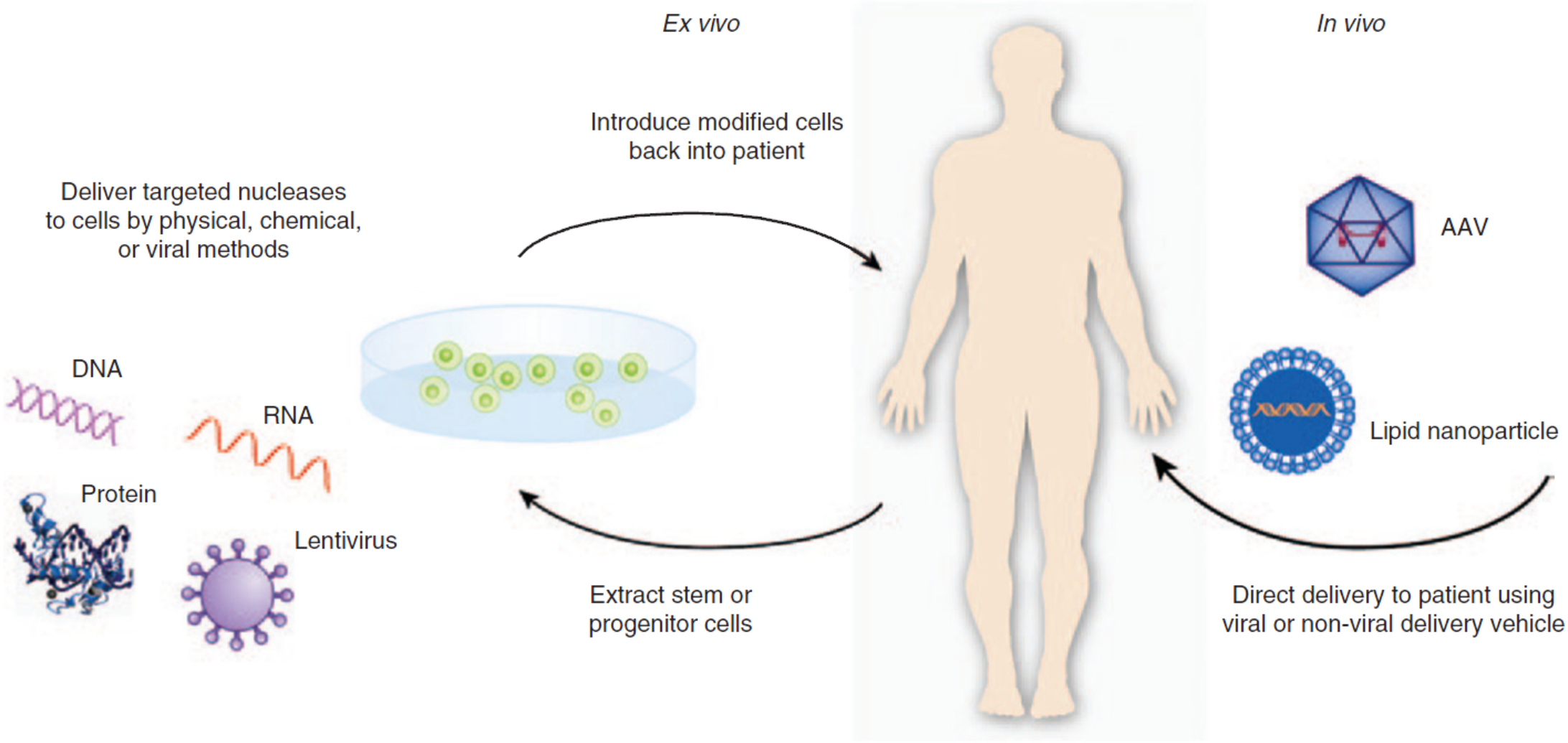
crRNA + tracrRNA = **gRNA**

Hsu PD, *et al.*, Cell, 2014

Gene-editing application

- Antivirial strategies
- Cancer immunotherapy
- Duchenne Muscular Dystrophy (DMD)

Delivery of genome-editing tools

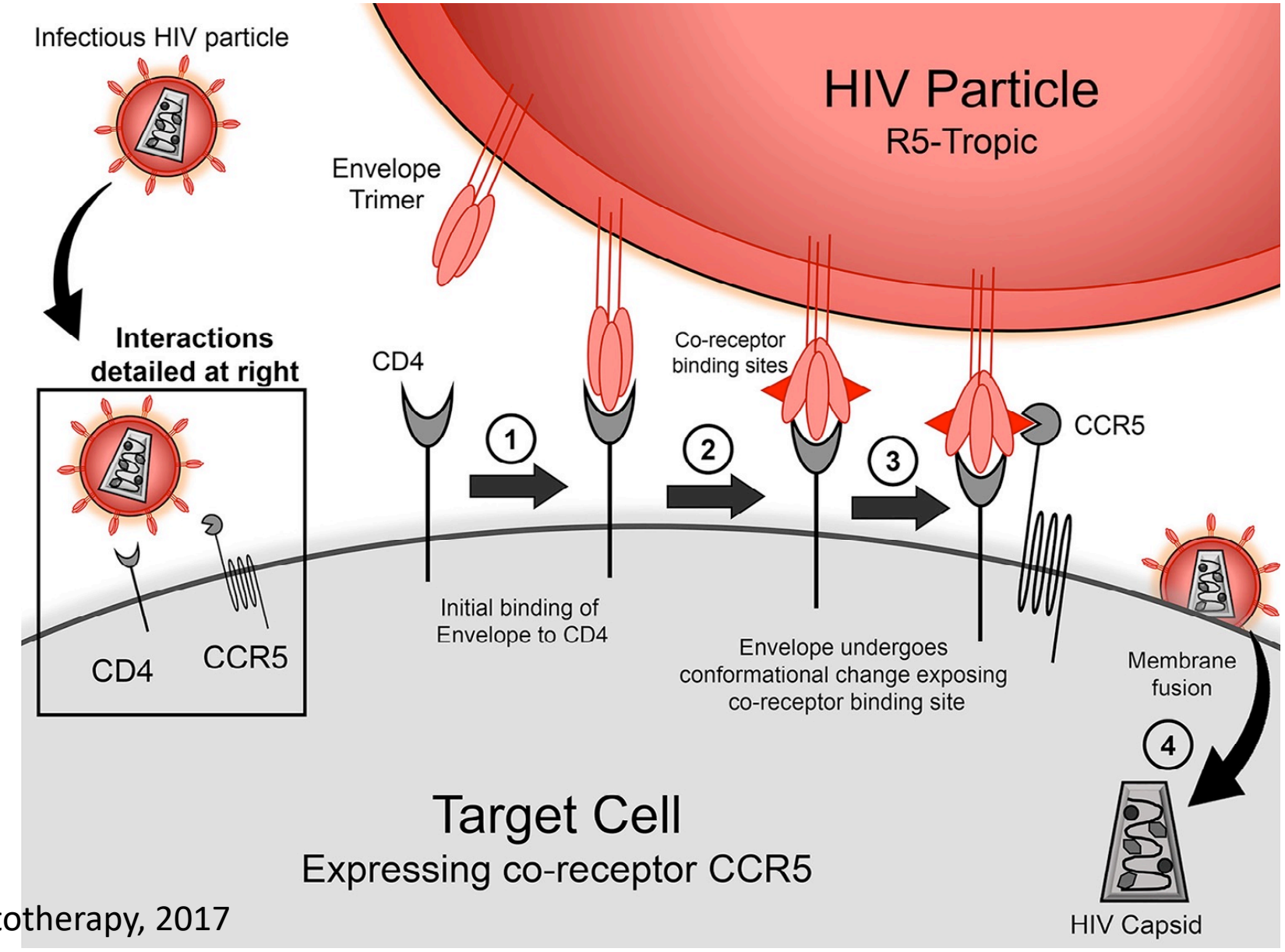


Maede ML & Gersbach CA, Molecular Therapy, 2016

Antivirial strategies

- the most advanced gene-editing strategy to date is the *ex vivo* modification of T cells to knock out the ***CCR5*** co-receptor used for primary **HIV infection**

Interactions between HIV particle and cell surface receptors during virus entry



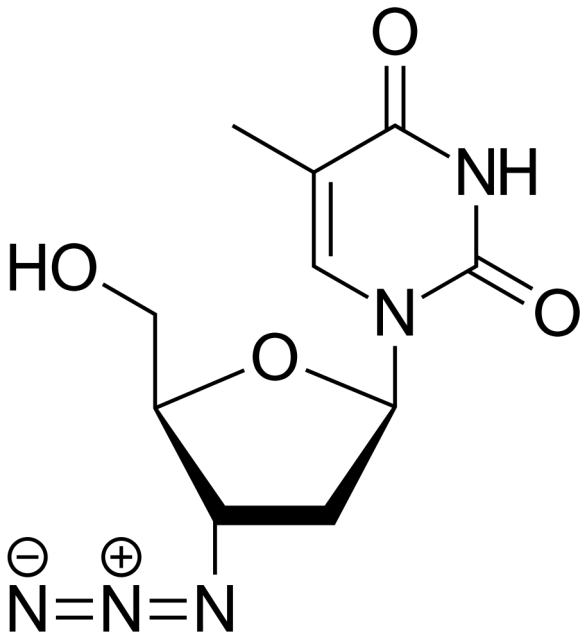
Haworth KG *et al*., Cytotherapy, 2017

Berlin Patient

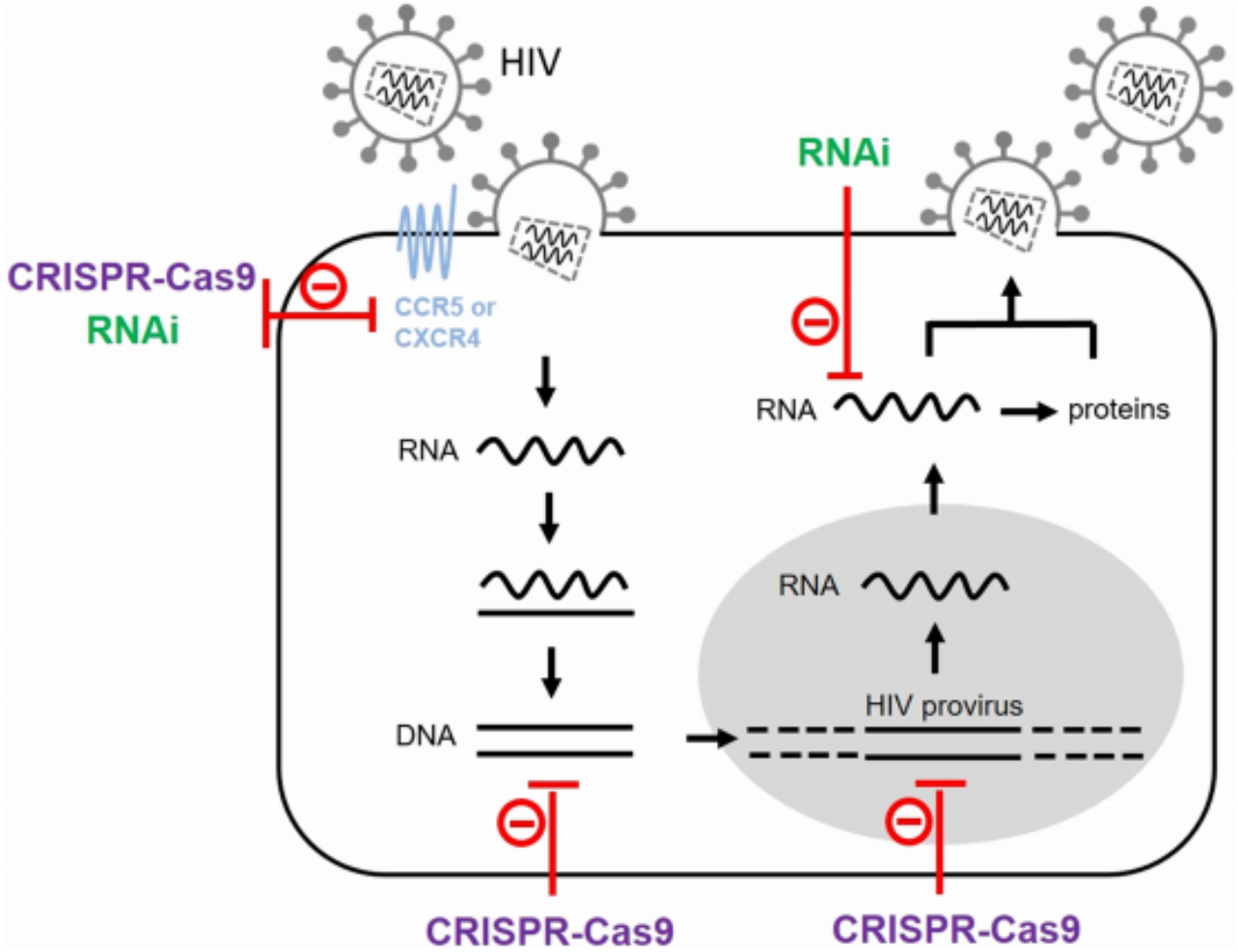
- First person cured of HIV infection (Timothy Ray Brown)
- Diagnosed with acute myeloid leukemia
- hematopoietic stem cell transplant
- Donor with the CCR5-Δ32 mutation
- CCR5-Δ32 – inactivates gene

HIV therapy

- combination antiretroviral therapy (**cART**)
- e.g. zidovudine (AZT) - thymidine analogue; blocks HIV's reverse transcriptase



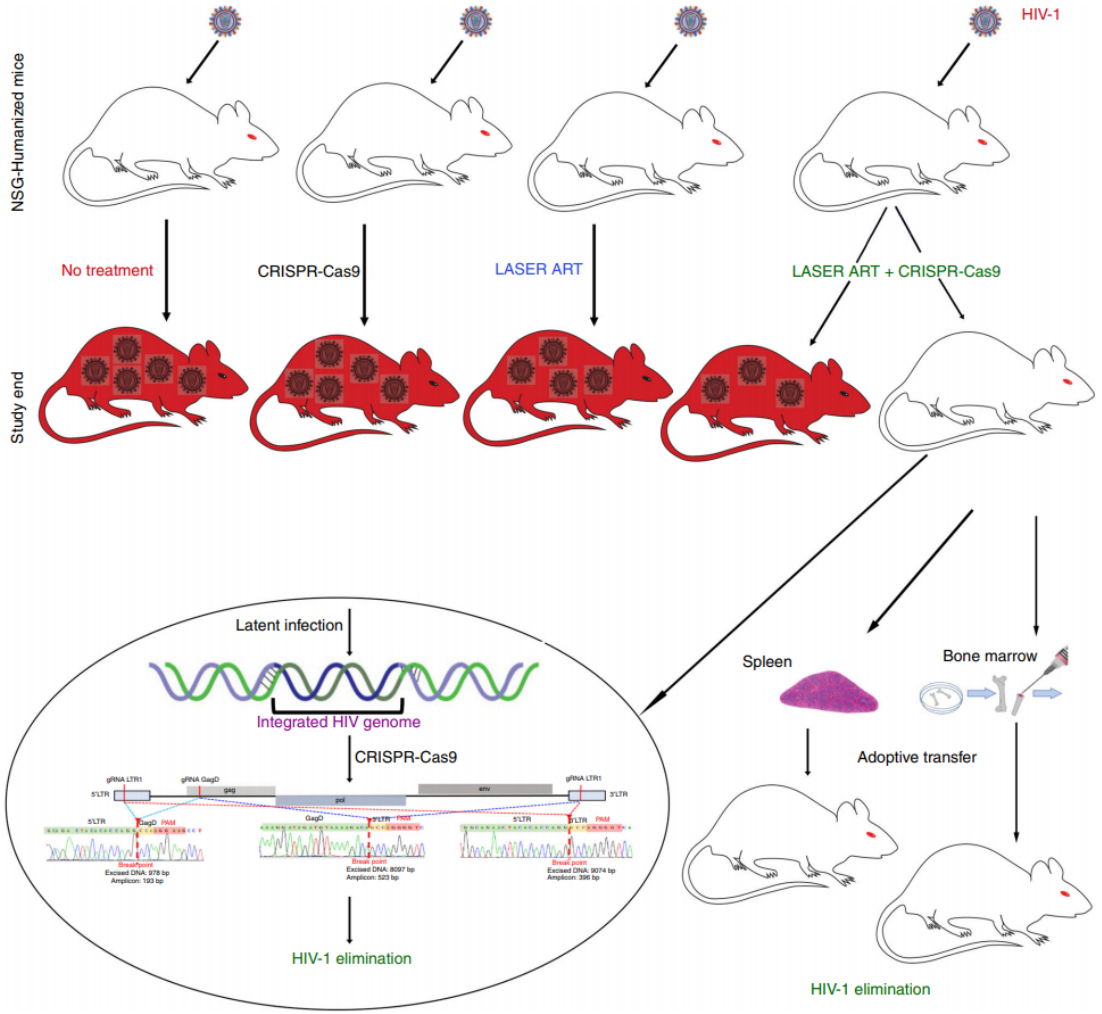
HIV therapy – future perspectives



Herrera-Carrillo E, *et al*., Briefings in Functional Genomics, 2019

HIV therapy – future perspectives

long-acting slow-effective release antiviral therapy (LASER ART)



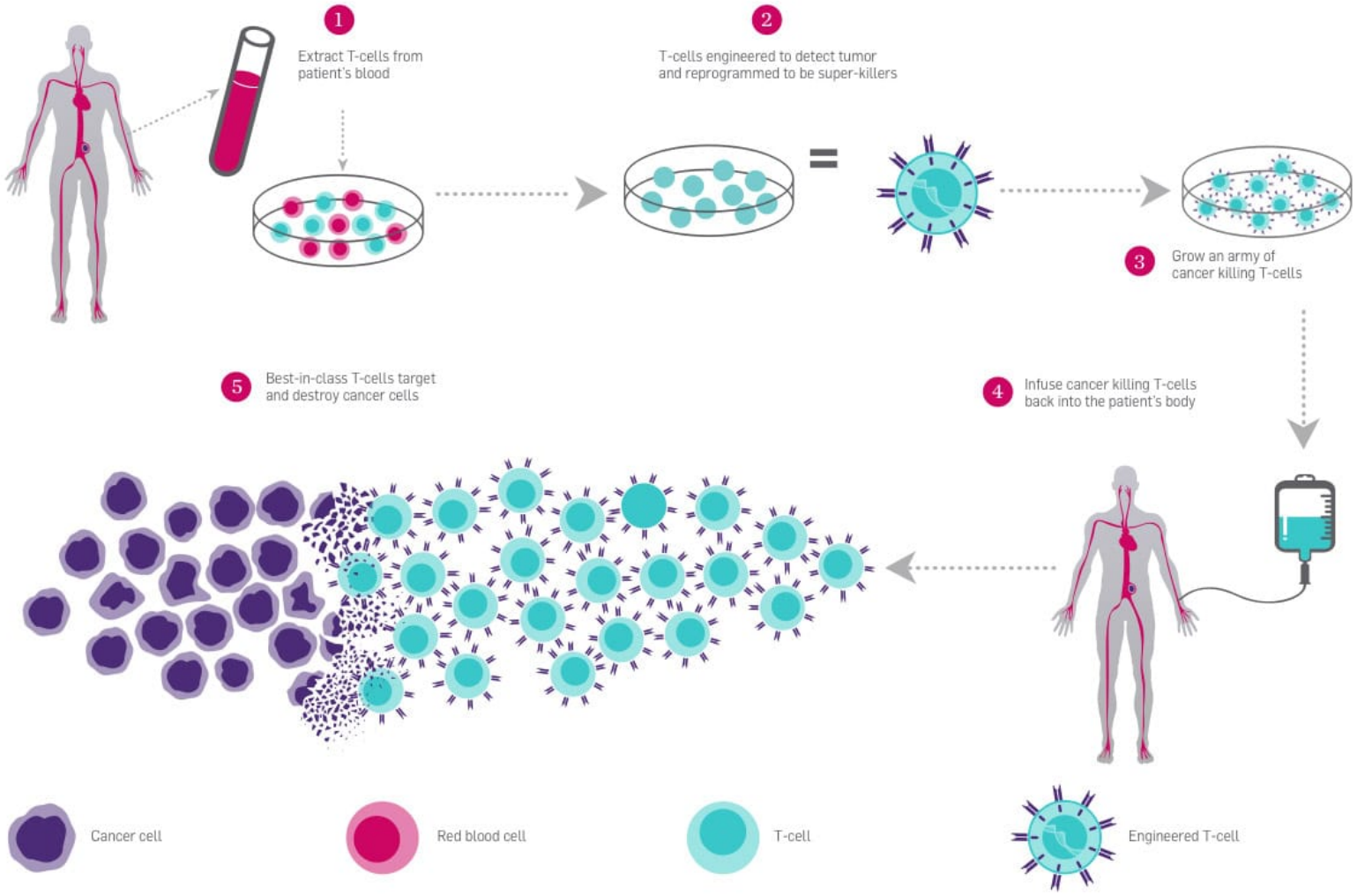
Dash PK, *et al*., Nature Communications, 2019

Cancer immunotherapy

Cancer immunotherapy

Active	Passive
targets tumor cells via the immune system e.g. cancer vaccines **CAR-T cell** targeted antibody therapies	enhances the ability of the immune system to attack cancer cells e.g. checkpoint inhibitors cytokines

Cancer immunotherapy – strategy

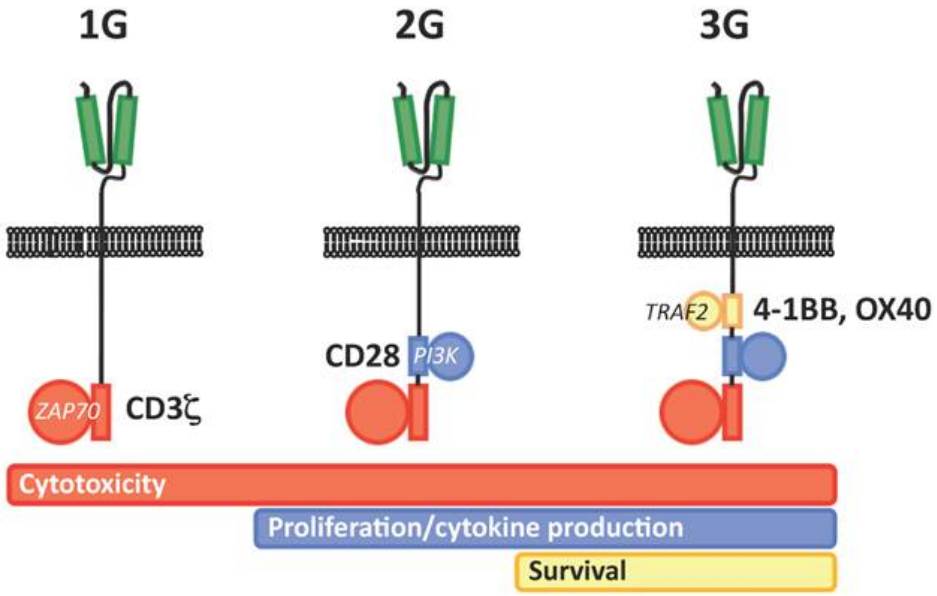


https://synbiobeta.com/crispr-clinical-trials-a-2019-update/

Cancer immunotherapy – CAR-T cells

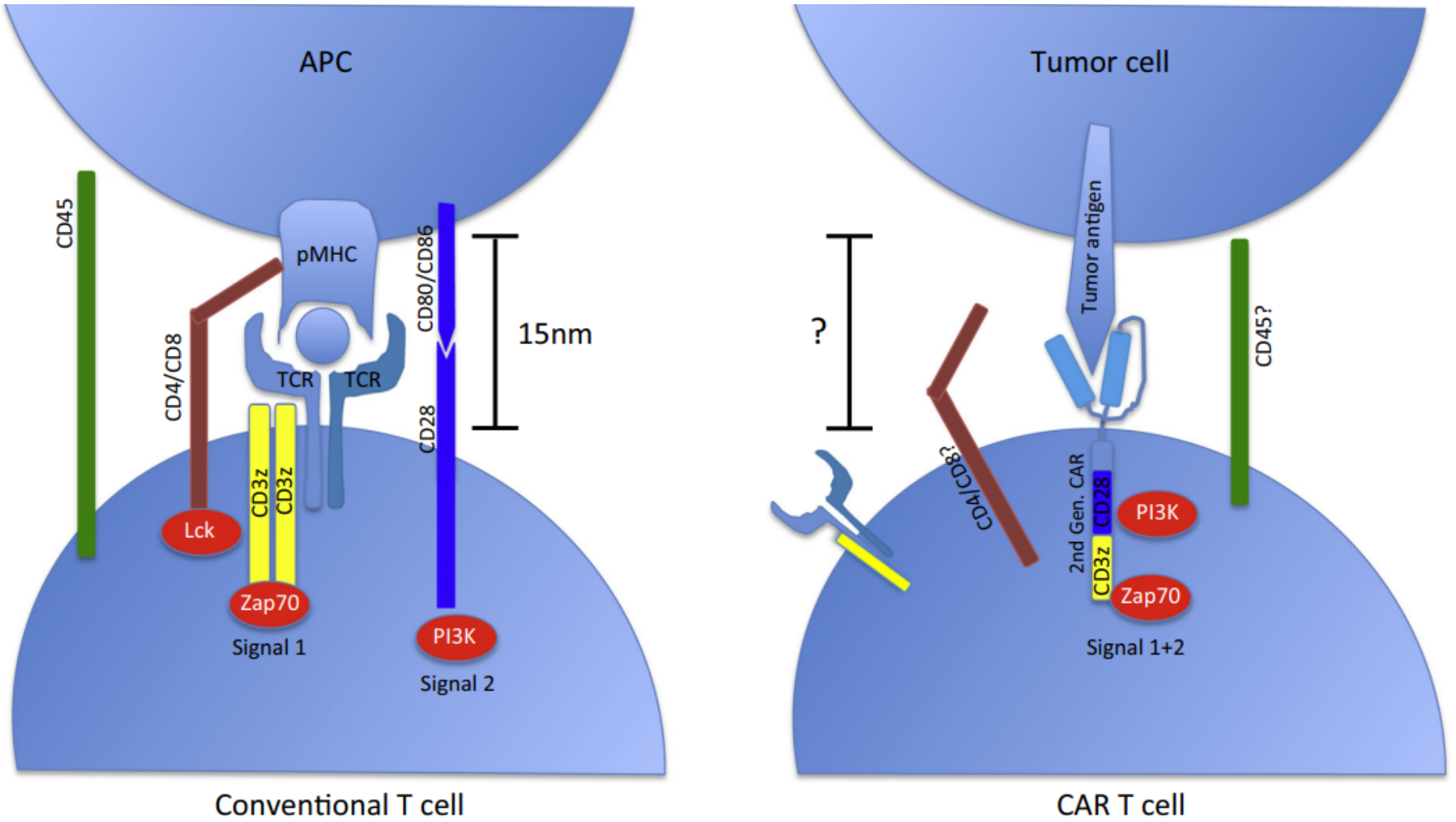
Chimeric antigen receptor T cells (also known as **CAR-T cells**)

Chimeric -> antigen binding + T-cell activating functions



https://upload.wikimedia.org/wikipedia/commons/a/ae/Depiction_of_3_generations_of_CARs.jpg

Cancer immunotherapy – CAR-T cells



Srivastava S *et al.*, Trends in Immunology, 2015

Cancer immunotherapy

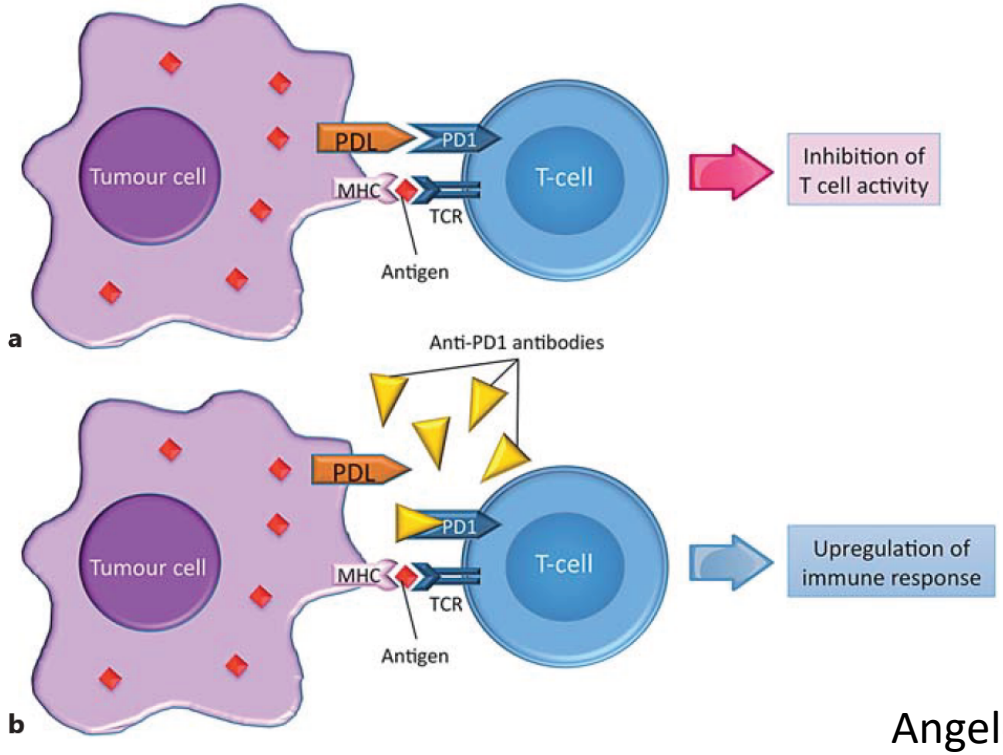
Adoptive T-cell immunotherapy, in which autologous T-cells are engineered to attack cancer antigens *ex vivo* and transferred back to the patient, has been impressively successful at treating some cases of **leukemia (targeted the antigene CD19), lymphoma,** and **melanoma**.

Cancer immunotherapy – challenges

- need to use autologous cells to avoid immune rejection
- the inhibition of T-cell effector functions by the expression of checkpoint inhibitors on the surface of tumor cells.

Cancer immunotherapy – challenges

- need to use autologous cells to avoid immune rejection
- the inhibition of T-cell effector functions by the expression of checkpoint inhibitors on the surface of tumor cells.

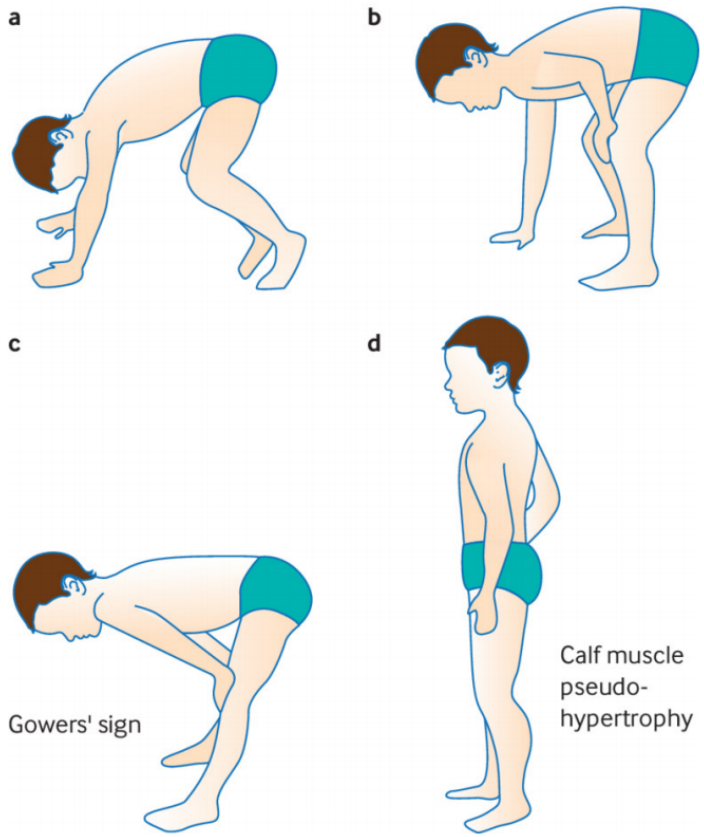


Angelousi A, *et al.*, Neuroendocrinology, 2018

Duchenne Muscular Dystrophy (DMD)

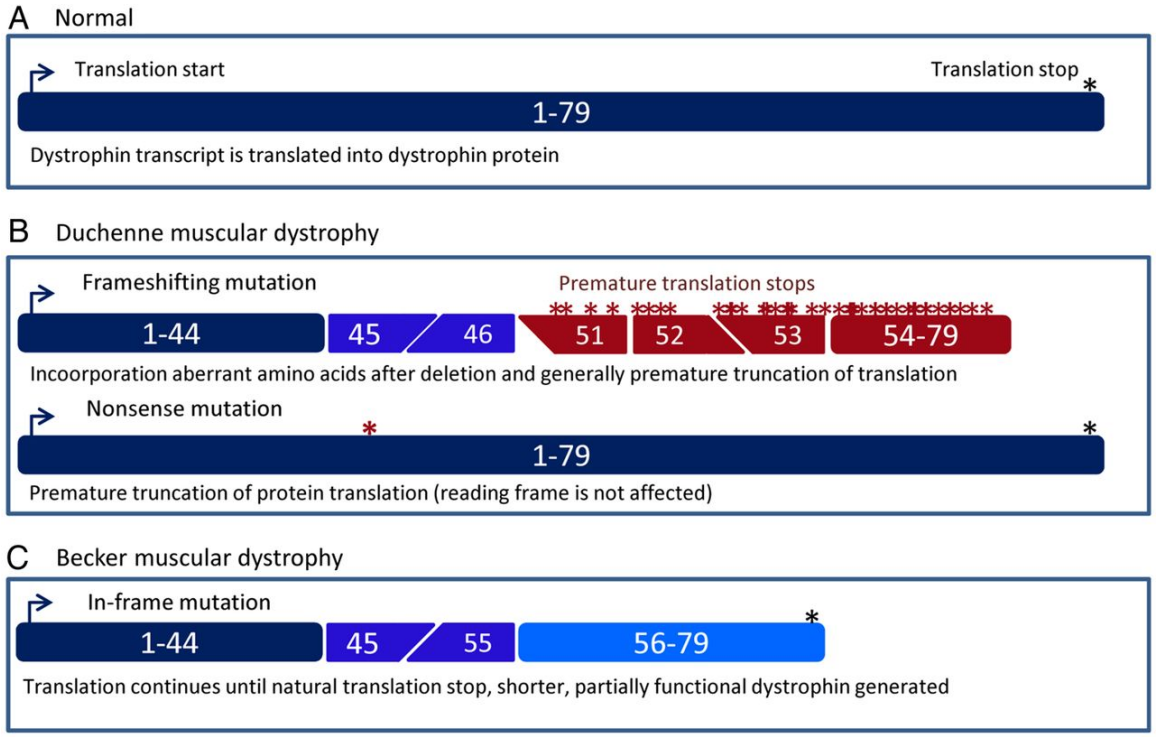
Duchenne Muscular Dystrophy (DMD)

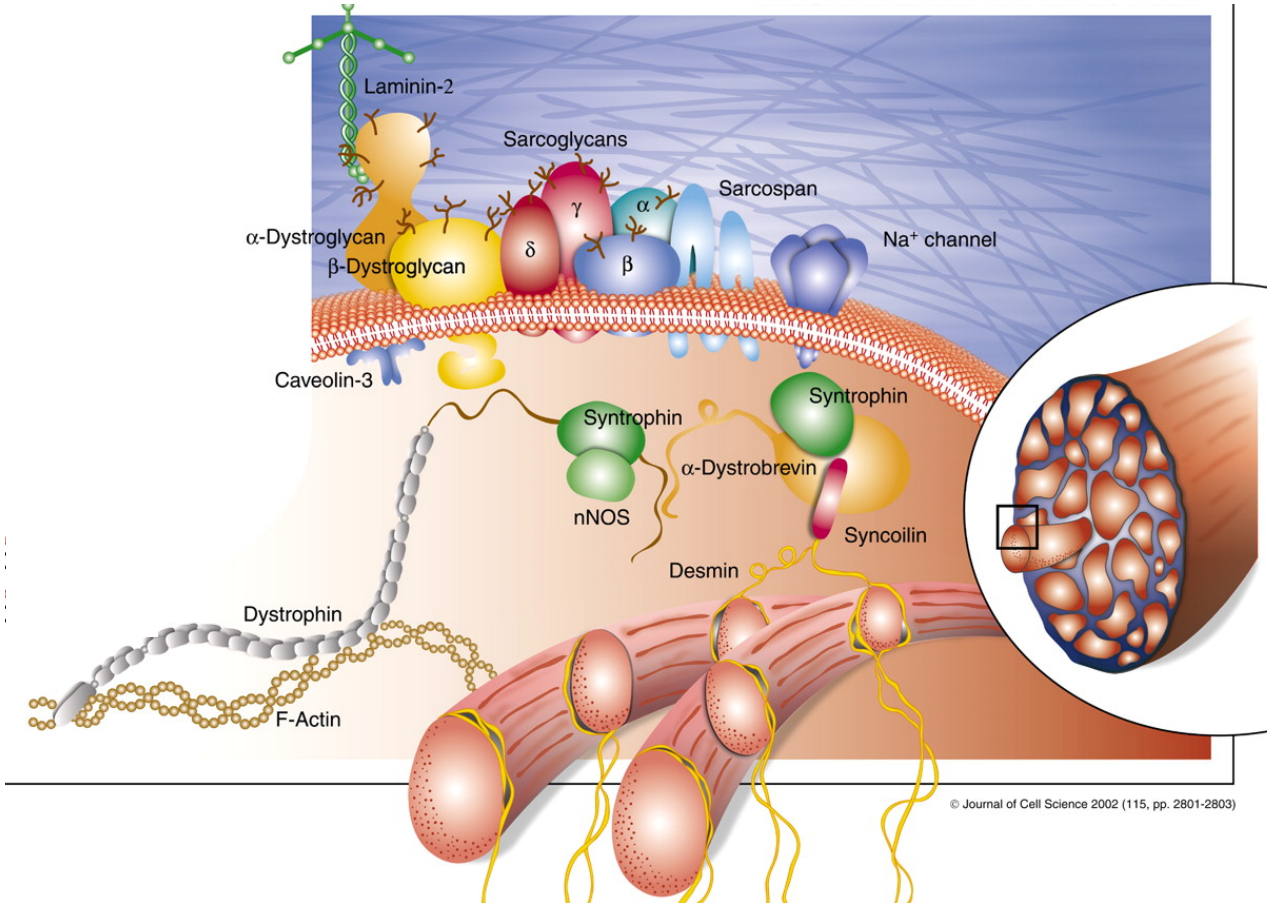
- 1:3500 male births
- Boys die in their 20s, due to respiratory or cardiac complications
- Symptoms:
  - Muscle weakening and wasting
  - Difficulties with: walking, running, climbing stairs, raising from the ground
  - Bulky calf muscles



Fox H, *et al.*, BMJ, 2020

Duchenne Muscular Dystrophy (DMD)

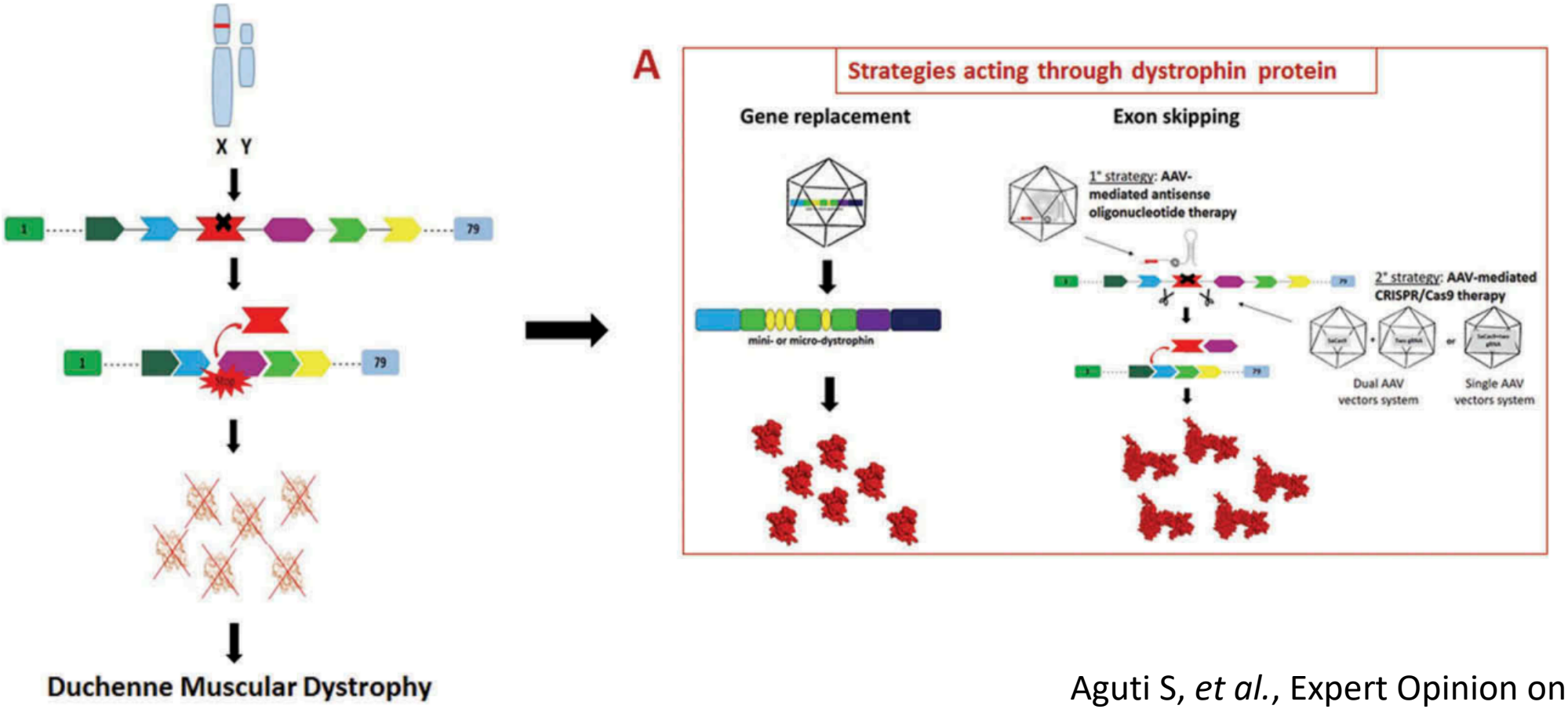




Aartsma-Rus A, *et al.*, Journal of Medical Genetics, 2015

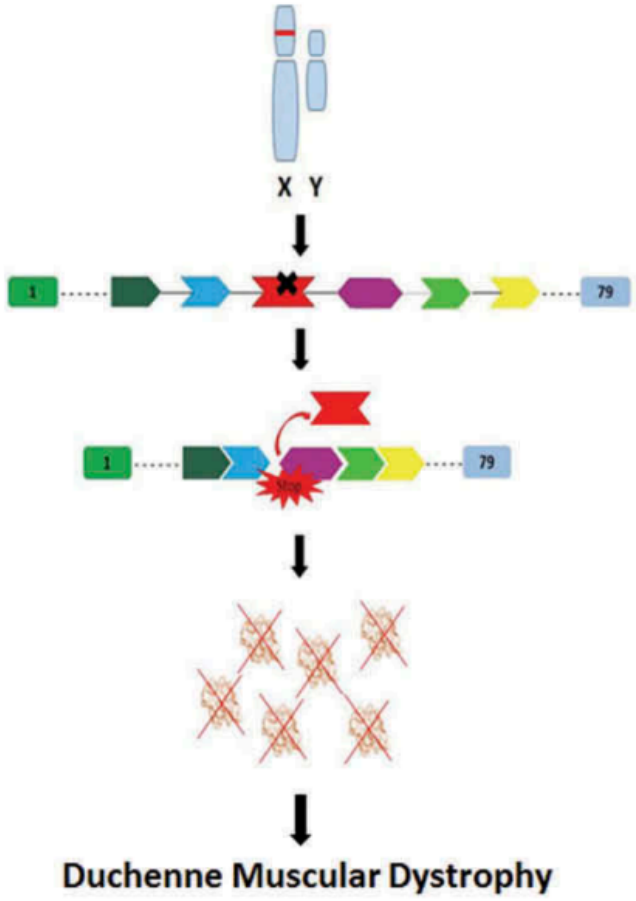
Ehmsen J, *et al.*, Journal of Cell Science, 2002

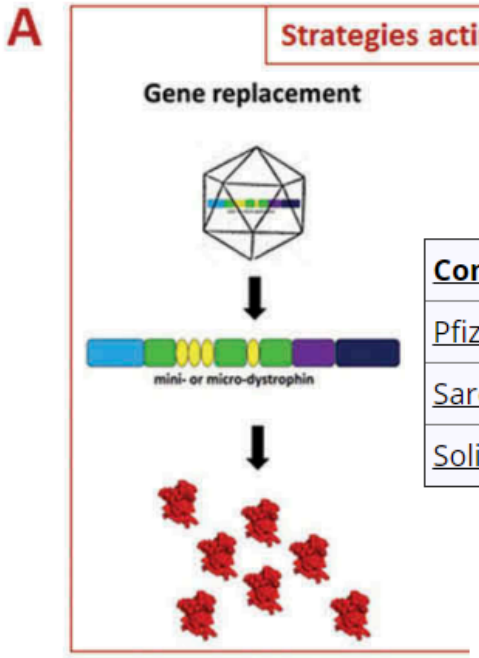
DMD – gene therapy



Aguti S, *et al.*, Expert Opinion on Biological Therapy, 2018

DMD – gene therapy





Company	Trial	Phase	Location	Update
Pfizer	PF-06939926	2	US	June 2019
Sarepta Therapeutics	EMBARK - SRP-9001	1/2a	US	June 2020
Solid Biosciences	IGNITE DMD - SGT-01	1/2	US	July 2020

Aguti S, *et al.*, Expert Opinion on Biological Therapy, 2018

CRISPR-mediated Genome Editing Restores Dystrophin Expression and Function in *mdx* Mice

Li Xu[1], Ki Ho Park[1], Lixia Zhao[1], Jing Xu[1], Mona El Refaey[1], Yandi Gao[1], Hua Zhu[1], Jianjie Ma[1] and Renzhi Han[1]

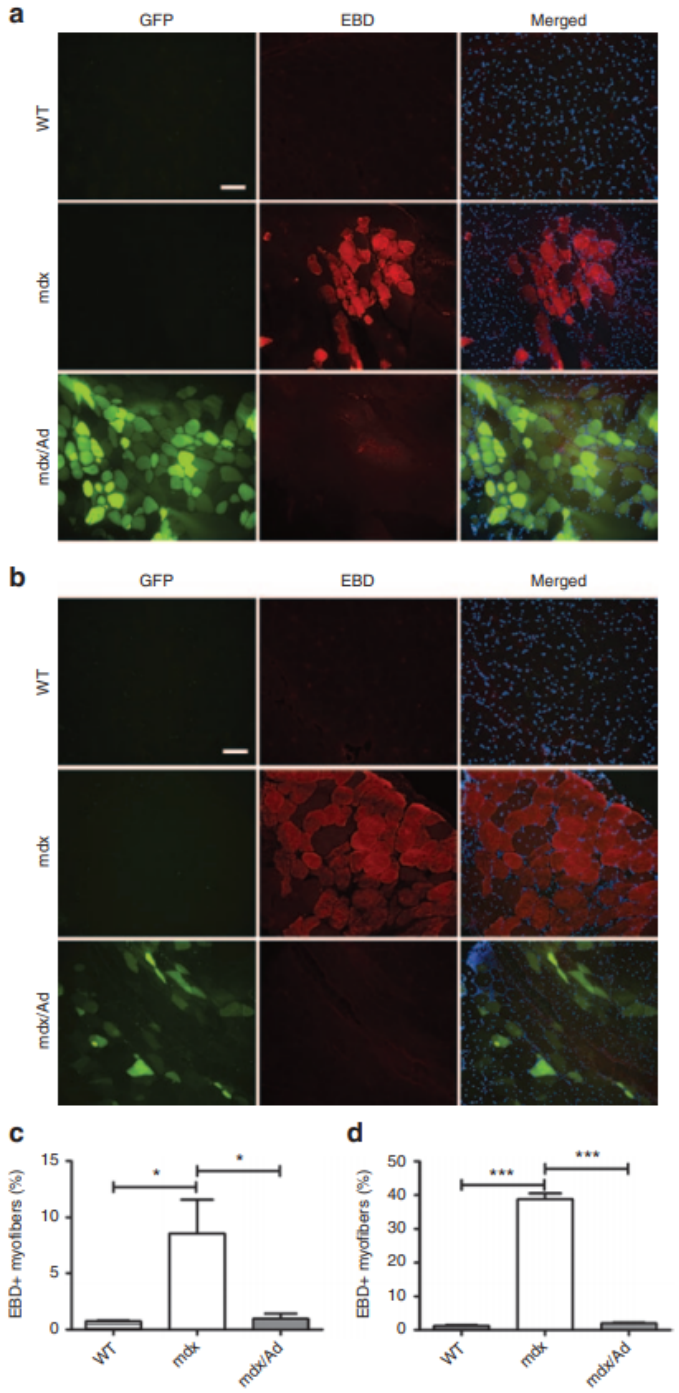
[1]*Department of Surgery, Davis Heart and Lung Research Institute, Biomedical Sciences Graduate Program, Biophysics Graduate Program, The Ohio State University Wexner Medical Center, Columbus, Ohio, United States*

Duchenne muscular dystrophy (DMD) is a degenerative muscle disease caused by genetic mutations that lead to the disruption of dystrophin in muscle fibers. There is no curative treatment for this devastating disease. Clustered regularly interspaced short palindromic repeat/Cas9 (CRISPR/Cas9) has emerged as a powerful tool for genetic manipulation and potential therapy. Here we demonstrate that CRIPSR-mediated genome editing efficiently excised a 23-kb genomic region on the X-chromosome covering the mutant exon 23 in a mouse model of DMD, and restored dystrophin expression and the dystrophin-glycoprotein complex at the sarcolemma of skeletal muscles in live *mdx* mice. Electroporation-mediated transfection of the Cas9/gRNA constructs in the skeletal muscles of *mdx* mice normalized the calcium sparks in response to osmotic shock. Adenovirus-mediated transduction of Cas9/gRNA greatly reduced the Evans blue dye uptake of skeletal muscles at rest and after downhill treadmill running. This study provides proof evidence for permanent gene correction in DMD.

Received 20 August 2015; accepted 5 October 2015; advance online publication 5 January 2016. doi:10.1038/mt.2015.192

CRISPR-mediated Genome Editing Restores Dystrophin Expression and Function in *mdx* Mice

Evans blue dye (EBD) is a reliable *in vivo* marker of myofiber damage



Bioethics

Chinese scientists are creating CRISPR babies – Lulu and Nana

They planned to eliminate a gene called **CCR5** in hopes of rendering the offspring resistant to HIV, smallpox, and cholera.

’’The birth of the first genetically tailored humans would be a stunning medical achievement, for both He and China. **But it will prove controversial, too**.

Where some see a new form of medicine that eliminates genetic disease, others see a slippery slope to enhancements, designer babies, and a new form of eugenics.”

https://www.technologyreview.com/s/612458/exclusive-chinese-scientists-are-creating-crispr-babies/

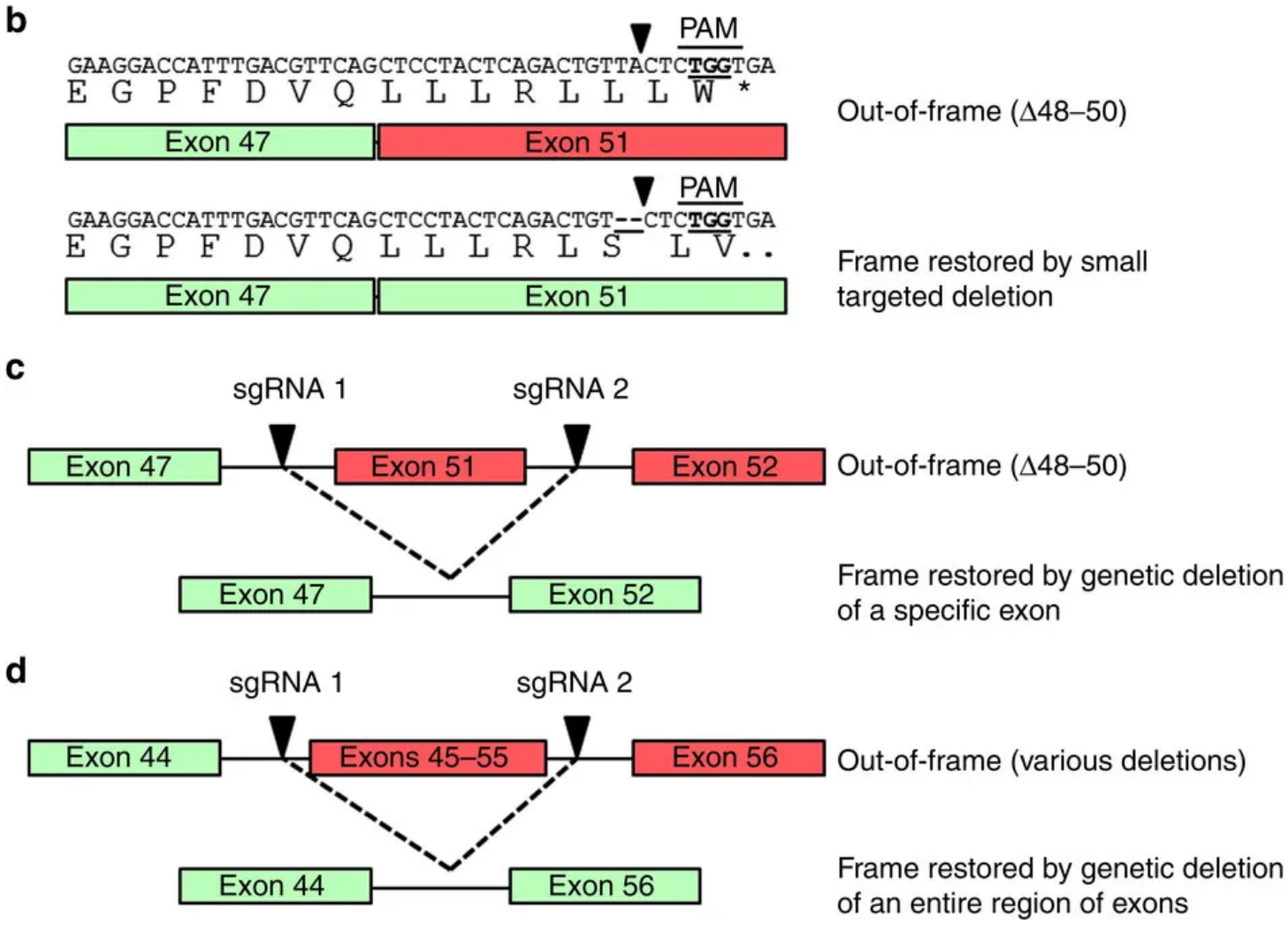
Table I. Clinical trials using CCR5 gene therapy.

Clinical trial ID	Status	Sponsor	Transplanted cell population	CCR5 modification method	Starting date	Ending date
NCT00569985	Ongoing	City of Hope Medical Center	Autologous HSPCs	Lentiviral CCR5 shRNA	June 2007	July 2017
NCT01961063	Recruiting	City of Hope Medical Center	Autologous HSPCs	Lentiviral CCR5 shRNA	June 2014	June 2031
NCT02337985	Recruiting	City of Hope Medical Center	Autologous HSPCs	Lentiviral CCR5 shRNA	August 2015	March 2018
NCT02343666	Recruiting	Fred Hutchinson Cancer Research Center	Autologous HSPCs	Lentiviral CCR5 shRNA	August 2016	August 2020
NCT02797470	Recruiting	AIDS Malignancy Consortium	Autologous HSPCs	Lentiviral CCR5 shRNA	May 2016	September 2019
NCT02378922	Recruiting	Fred Hutchinson Cancer Research Center	Autologous HSPCs	Lentiviral CCR5 shRNA	June 2016	June 2019
NCT01734850	Ongoing	Calimmune, Inc.	Autologous mobilized HSPCs and T cells	Lentiviral CCR5 shRNA	April 2013	October 2017
NCT00842634	Completed	University of Pennsylvania	Autologous T cells	ZFN modified (Adenovirus)	January 2009	January 2013
NCT01543152	Ongoing	Sangamo Therapeutics	Autologous T cells	ZFN modified (Adenovirus)	December 2011	September 2017
NCT01044654	Completed	Sangamo Therapeutics	Autologous T cells	ZFN modified (Adenovirus)	December 2009	December 2014
NCT01252641	Completed	Sangamo Therapeutics	Autologous T cells	ZFN modified (Adenovirus)	November 2010	May 2015
NCT02225665	Ongoing	Sangamo Therapeutics	Autologous T cells	ZFN modified (Adenovirus)	August 2014	June 2018
NCT02388594	Recruiting	University of Pennsylvania	Autologous T cells	ZFN modified (Adenovirus)	Febuary 2015	October 2017
NCT01153646	Terminated	City of Hope Medical Center	Autologous T cells	Lentiviral CCR5 RNAi	April 2010	January 2011
NCT02500849	Recruiting	City of Hope Medical Center	Autologous mobilized HSPCs	ZFN modified (Adenovirus)	July 2015	July 2018

Data obtained from the publically available National Institutes of Health database: www.clinicaltrials.gov.
ID, identification; shRNA, short hairpin RNA.

Haworth KG *et al.*, Cytotherapy, 2017

Duchenne Muscular Dystrophy (DMD)



Ousterout DG, *et al.*, Nature Communication, 2015